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Publication date:
2017

Document Version
Publisher's PDF, also known as Version of record

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PhD Thesis: Rachel Amanda Hickman
14th February 2017

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Preface

This PhD thesis was prepared at Novo Nordisk Foundation Center for Biosustainability at the Technical University of Denmark (DTU) in fulfillment of the requirements for the PhD degree. Research work was performed both at the Centre for Systems Microbiology (2014-2015) and at the Novo Nordisk Foundation Center for Biosustainability (2015-2017).

The work was carried out under the supervision of Professor Morten Sommer (Supervisor) and Professor Søren Molin (co-supervisor) and funded by a PhD stipend from DTU.

14th February 2017

Rachel Amanda Hickman

Acknowledgements

There are many people I would like for making my time through my PhD enjoyable and do-able.

Firstly, and most importantly, I would like to thank **Morten** for giving me this opportunity to this PhD and allowing me to research cool and exciting science and pushing me outside my comfort zone and **Søren** for his support when I was overwhelmed.

I would also like to thank **Christian** for guiding me and supervising my as a new lost PhD student and **Andreas** his partner in crime that has help on many occasions, with R and the Danish abstract within this PhD thesis! I would also like to thank **Lejla** for providing DNA extracts that formed the pilot study for the second manuscript, **Helle** for her depth reading and guidance of this manuscript and **Jakob** for his tolerance and help in this project. I would also like to thank **Lumeng** for helping on the crazy FACs project and tips on Pseudomonas aeruginosa PCR!

There are so many people from the Sommer lab group that I'm also grateful for such as **Gitte** for group organization and oracle of the tem system, **Mari** for finding obscure lab items, PhD comradery from the Sommer PhD office (**Ruben**, **Micheal**, **Kira**, **Gonzalo** and un-officially **Christian N**) and other members of the **Sommer** lab group both past and present as well as other **CFB** members.

Finally, I'm also very grateful for the support from my family and my partner Erik and would like to dedicate this PhD thesis to them as without them it would be impossible.

Tusind tak!

Abstract

Bacteria have existed on earth for 3.5 billion years and their ability to evolve has allowed for their survival in almost all global niches. Bacteria evolve and adapt easily due to their short generation times, plastic genomes, acquisition (external) DNA and their ability to form protective bacterial communities i.e. biofilms or dormant metabolic states.

Antibiotic drugs are currently our best medicine to treat (against) bacterial pathogens due to antibiotics unique properties of being small molecules that are soluble and act systemically. These qualities allow for many modern medical procedures to occur due to antibiotics preventative/ prophylactic and therapeutic qualities.

Despite bacterial antibiotic resistance mechanisms always being present in nature, the overuse and misuse of antibiotics by humans are accelerating the rise and dissemination of bacterial antibiotic resistance. Bacterial antibiotic resistance is global threat to public health; especially because of lack of new drugs. It has been highlighted that understanding antibiotic resistance by further elucidating mechanisms of evolution, molecular mechanisms of action and reservoirs of resistance are essential Therefore, the work involved in this PhD thesis, examines the evolution of antibiotic resistance in bacterial populations.

Two main studies were performed: the first to elucidate the molecular mechanisms of collateral sensitive drug pairs and collateral resistance drug pairs in adaptation of *Escherichia coli* populations; and the second exploring mutant variant dynamics in cystic fibrosis lung, by analyzing sputum samples from chronic carriers of *Pseudomonas aeruginosa* undergoing antibiotic treatment.

Both studies explore the trajectories of antibiotic resistance within bacterial populations: the first study by exploring antibiotic resistance loci, and the in the second by whole-gene sequencing. The desired outcome from both studies is to find methods to use antibiotic therapy more rationally to treat infection efficiently and effectively whilst reducing the evolution of antibiotic resistance.

Dansk resumé

Bakterier har eksisteret på jorden i 3,5 millioner år, og i den tid har deres evne til at udvikle sig tilladt deres overlevelse i stort set alle tænkelige nicher. Bakterier udvikles og tilpasses let på grund af deres korte generationstider, plastiske genomer, erhvervelsen af eksternt DNA samt deres evne til at danne beskyttende bakterielle samfund dvs. biofilm.

Antibiotika er i øjeblikket vores bedste medicin til behandling af bakterielle patogener på grund af dets opløselighed systemiske virkning. Disse kvaliteter er vitale for mange moderne medicinske procedurer på grund af antibiotikas profylaktiske og terapeutiske egenskaber.

På trods af at antibiotika resistensmekanismer altid har været til stede i naturen, har samfundets overforbrug og misbrug af antibiotika fremskyndet stigningen og formidling af bakteriel antibiotikaresistens. Bakteriel antibiotikaresistens er en global trussel mod den offentlige sundhed; især på grund af manglen på nye lægemidler. En grundigere forståelse af antibiotikaresistens ved yderligere at belyse evolutionsmekanismer, molekylære virkningsmekanismer og reservoirer er afgørende. Derfor belyser dette PhD-arbejde udviklingen af resistens i bakterielle.

To hovedundersøgelser blev udført: den første bestod i, yderligere at belyse de molekylære mekanismer i antbiotikapar som indbyrdes forstærker eller svækker resistensevolution i *Escherichia coli* populationer. Det andet studie udforsker mutantvariant dynamik i CF lunge-prøver fra kronisk *Pseudomonas aeruginosa* inficerede cystisk fibrose patienter under igangværende antibiotisk behandling på hospitalet. Begge undersøgelser udforsker udviklingsveje for antibiotikaresistens i bakterielle populationer: det første studie ved at udforske kendte antibiotikaresistensmutationer i genomet, og det andet ved sekventering af hele gener involveret i resistens. Det ønskede resultat fra begge undersøgelser er at finde metoder til at bruge antibiotika, der både behandler patogen effektivt og samtidig hindre udviklingen af antibiotikaresistens.

List of Publications

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. **R.A. Hickman,** C. Munck and M.O.A Sommer. Time-resolved tracking of mutations reveals strong clonal interference during antimicrobial adaptive of *Escherichia coli* to single and drug pairs. (Currently in interactive review to Frontiers in Microbiology)

II. **R.A. Hickman**, J. Frimodt-Møller, E. Rossi, H.K. Johansen, S. Mølin and M.O.A. Sommer. Direct sequencing of *Pseudomonas aeruginosa* from sputum of cystic fibrosis patients undergoing antimicrobial therapy. (Manuscript in preparation).

Contents

Abbreviations

ALE	Adaptive Laboratory Evolution
AMK	Amikacin
CHL	Chloramphenicol
CIP	Ciprofloxacin
EUCAST	The European committee on antimicrobial susceptibility testing
HGT	Horizontal gene transfer
IC	Inhibitory Concentration
I	Intermediate antimicrobial susceptible type
INDELs	Insertion or deletions sequences
MDR	Multi-drug resistance
NGS	Next generation sequencing
ROS	Reactive oxygen species
R	Resistant antimicrobial susceptible type
SNPs	Single nucleotide polymorphisms
S	Susceptible antimicrobial susceptible type
WGS	Whole genome sequencing
WHO	World Health Organization

Outline of PhD Thesis

This thesis is composed of 5 chapters: Bacterial Evolution, Antibiotic drugs, Profiling and Exploring Drug Treatment and Resistance, Applications of Profiling of Drug Treatment and Resistance, and Concluding Remarks and Future Perspectives.

Each chapter composes of a short introduction, to provide the overview of the importance of the chapter, followed by sub-chapters. Within each of the sub-chapter's ideas, theories and links to scientific literature are given and then summarized with how this applies to the to the research work done in this PhD time-period.

Bacterial Evolution

Within most natural environments a diverse consortia of bacteria species exist. Following the evolutionary laws of nature, bacteria have adapted to a variety of physiological demands such as pH [1], osmotic pressures [2], temperature [3], nutrient availability [4] and chemical perturbations like disinfectants [5], metal ions [6] and antibiotics [7]. Bacteria have several evolutionary constraints e.g. asexual reproduction that limits chromosomal genomic recombination and a high codon bias genome of ca. 90% [8]. Despite these constraints, bacteria can easily and rapidly evolve due to their short generation time (e.g. *E. coli* K-12 sub-strain MG1655 in Luria-Bertani broth at 37^{o}C has a doubling time during steady-state of 20 minutes [9]), their large population size capability (e.g. up to 2x10^{9} CFU/mL [10]), their rate of spontaneous mutation (e.g. ~ 0.03 per genome per replication [11]), and their genetic exchange between taxa [12]). These advantageous characteristics promote both anagenesis, which allows evolution within the bacterial species population, and cladogenesis, which allows new bacterial species to develop in different biological niches [13].

Processes that allow bacterial genomic modification

To accomplish these abilities, bacteria rely on mechanisms to acquire novel genetic information and manipulate their genome. This achieved by three main processes: bacterial transduction, transformation and *de novo* mutations. The first two processes occur by horizontal gene transfer (HGT), which unlike other forms of life defy species specific boundaries. Therefore, a microbial habitat can quickly possess abilities to defy the abilities of given antimicrobials, metal ions and disinfectants. An infamous example of this is the CTX-M-15 plasmid that confers resistance to antimicrobials, metal ions and biocides [14]. Furthermore, it is easily disseminated in Enterobacteriaceae and as a consequence several clinical outbreaks have occurred [15–17]. *De novo* mutations, however, occur intrinsically within a cell and if remain uncorrected by cellular correction systems these mutations will be passed on to its future daughter cells. As mentioned earlier HGT resistance mechanisms defy species specific boundaries. It could therefore be easy to assume that more scientific effort should be concentrated on HGT resistance

mechanism rather than *de-novo* mutational resistance mechanisms, however both play an integral role in adaptation evolution. For some bacterial species – such as *P. aeruginosa* in chronically infected cystic fibrosis patients [18,19] and *Mycobacterium tuberculosis* where HGT [20] – evolution is generated mainly or solely by the latter mechanism. Therefore, scientific research in both types of acquired resistance are important, especially when it comes to understanding bacterial evolution in a wide range of organisms and perturbations.

With the research involved in this PhD doctorate, I have looked into the types of *de novo* mutations that occur in the bacterial population. *De novo* mutations are a result of genomic instability. Genomic instability is induced by the constant threat that bacterial cells are exposed to, such as operations of their DNA replication and repair systems, mobile elements, phages, chemical entitles and environmental factors [21]. *De novo* mutations manifest themselves as point mutations, genomic rearrangements or translocations, and can be inherited to future prodigy if remained un-corrected by cellular repair systems. Point mutations, or single nucleotide changes (SNPs), can induce three types of amino acid coding changes. The effects of these changes are silent, when no effects are observed to coding amino acid, missense, where a different amino acid is coded for and is also referred to as a non-synonymous change, and nonsense mutations, which encodes for a stop-codon that terminates the transcription of the RNA prematurely. Genomic rearrangements are induced by insertion or deletions sequences (INDELs) or the combination thereof, amplifications, or translocations. The result of INDELs in the genome cause change to the encoded amino acid sequence of a transcribed mRNA strand by either the addition or subtraction of codons or by causing frame shifts to encode entirely different amino acids. The results of gene duplication are common in both bacteria and eukaryotes [22]. A possible model for gene duplication is the innovation-amplification-divergence model. In this model an ancestral gene has a weak secondary activity, and in a given favorable selection pressure a cell that duplicate this gene create a different gene from the ancestor by genetic modification [23]. From the research work performed during this PhD, my main focus was on missense and nonsense mutations SNPs, INDELs and possible gene duplication events that occur during antibiotic adaptation or during antibiotic treatment.

Evolutionary theory: Neo-Lamarckism versus Neo-Darwinism?

Genomic modifications which lead to bacterial phenotypes that are better suited to a given environment are essential for bacterial evolutionary development, but which evolution hypothesis that induce these effects is uncertain. Therefore, the theory that best answer the evolutionary development process is under scientific debate again, as the impact of understanding process that lead to genomic stability play an important role in curbing antibiotic resistance.

The two main theories are Neo-Lamarckism and Neo-Darwinism. The Neo-Lamarckism theory assumes that external environmental cues causes individuals to change their phenotype in response. Individuals that are successful in changing their phenotype to adapt will pass this on to their future progeny. The Neo-Darwinism theory follows that the basic unit of evolution is a population, where genomic variation occurs by random genetic drift and selection for given phenotypes is driven by environmental factors. Evidence for both theories has been produced by the use of next generation sequencing (NGS) and other typing methods. Direct evidence for bacterial Neo-Lamarckism can be found in the CRISPR-cas system, where integration of small segments of HGT DNA into specific loci allows for host defense (Koonin and Wolf 2009). Most *de-novo* antibiotic resistance is nonetheless assumed to be the result of Neo-Darwinism. This is seen in bacterial populations where contact with sub-lethal concentrations of antibiotics generates random mutants either by inducing direct effects (e.g. bleomycin inducing double-strand DNA breaks [24]) or in-direct effects (e.g. ampicillin downstream production of reactive oxygen species (ROS) that incudes DNA damage [25]). These mutations establish themselves in the population and can be fixed or lost in the bacterial population by clonal interference. However, a quasi-Lamarckism mechanism coupled with neo-Darwinian mechanism, permits bacterial cell to adapt to various environmental perturbations by allowing a regulation of genomic instability to occur followed by natural selection [26]. The quasi-Lamarckism mechanism was first suggested by Radman, circulated privately in 1970, where she states that "'SOS-replication' mechanism can be induced by a variety of mutagenic treatments, which cause inhibition of 'vegetative' DNA replication…This replication mechanism is an inaccurate, mutation-prone process" (Embedded in [27]). Interestingly many antibiotic drugs classes used in the experimental work of this PhD thesis also induce SOS- replication response. The drug classes that induced these effects are: aminoglycosides [28], chloramphenicol [29] and fluoroquinolones [30]. Therefore, it is interesting to note that, sub-lethal exposure to antibiotics can induce DNA damage and trigger genomic instability that therefore induced genetic diversity of microbial pathogens

(Shapiro 2015). Despite none of these quasi-larmarckian mechanisms being validated experimentally in this PhD thesis, the neo-Darwinian mutations I observe could also be a result of the antibiotics inducing genomic instability facilitated by quasi-larmarckian mechanisms. Therefore, this is something I would like to return to in the concluding remarks and future perspectives.

Bacterial population adaptations to novel environmental challenges

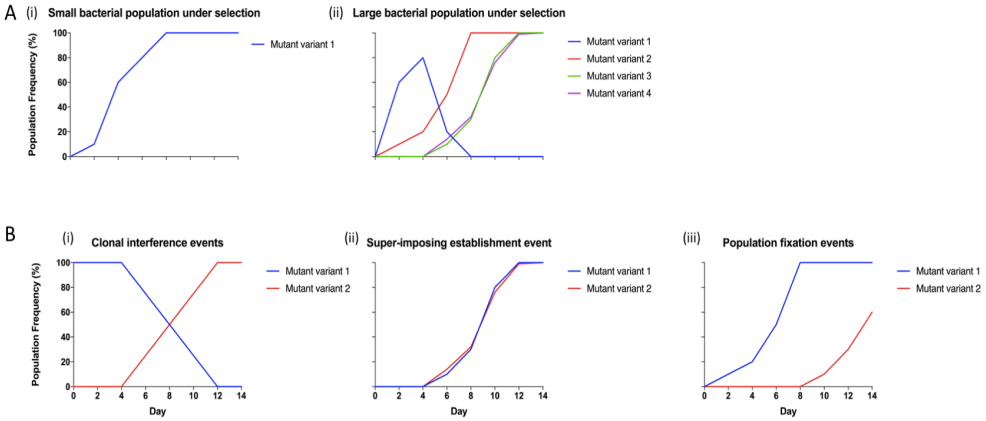
For bacteria populations to adapt to a new environmental challenge or perturbation, novel mutation cells must occur, reproduce in quantities as not to be lost by random genetic drift. This process is known as bacterial mutant allelic establishment, where a sub-population of this clone type develop within a population. If this mutant allelic sub-population is able to take over the population and dominate 100% of the population this is known as fixation.

Two variables govern a bacterial population's ability to survive a new environmental challenge these are: the range of beneficial mutations that are able to be produced in the population and the size of the population [31]. In a small bacterial population, the range of beneficial mutants is less, therefore that population has less likelihood of surviving is also less [32]. However, if a beneficial mutation allelic sub-population does arise and become established it has an almost certain probability of fixing in the population. This is opposite in larger population due to the range of beneficial mutations being higher (e.g. clonal divergence), so likelihood of surviving is also higher. However, the beneficial mutation allelic sub-population or clone has less chance of becoming fixed in the population. This is due to clonal interference, where two novel genotypes or more compete with each other to establish themselves within the population [33]. Different genotypes can occur in different genetic backgrounds within the population, therefore the large the bacterial population, the higher diversity in bacterial genetic background and novel beneficial mutational allelic types [34]. All these characteristics play an important role in evolution population mutagenic allelic dynamics, where periodic selection of various clones of mutant alleles occur. The overall result of clonal interference in a large population acts as a natural selection process that selects beneficial mutations that has the largest effect at the smallest fitness cost, known as periodic selection [35].

The work I presented in this PhD thesis explores the characteristics of evolution of bacterial population mutagenic allelic dynamics by time-resolved tracking known mutagenic loci in *in-vitro* antibiotic *E. coli* adaption (manu-

script I) or in known antibiotic genes of *P. aeruginosa* of chronically infected cystic fibrosis patients under going 14 days of antibiotic treatments (manuscript II). From these results, I see in the bacterial populations strong evidence of clonal divergence, clonal interference and periodic selection, however by the methods use I can not confirm the genetic backgrounds these beneficial mutations and can not confirm any historical contingency events. Interactions I see within my populations are summarized in (Fig 1).

Fig 1. Summary of generalized population mutant variant interactions (A) Population size effect (i) small bacterial population under selection, (ii) large bacterial population under selection. (B) Mutation variant interactions (i) clonal interference events, (ii) super-imposing establishment events, (iii) population fixation events.



Antibiotic drugs

The discovery and use of antibiotic drugs allowed the birth of modern medicine to occur by allowing medical procedures to be performed whilst reducing the risk of pathogenic bacteria. Antibiotic drugs are currently our best medicine to treat against bacterial pathogens due to antibiotics' unique properties of being small molecules that are soluble and act systemically [36]. Bacteria have a tremendous ability to evolve and develop genotypes to overcome antibiotic drug effects, and when this occurs in invading bacterial pathogens they cause mortality and morbidity. It is essential to understand where antibiotic drugs come from, what classes of drugs we have, how bacteria become resistant to these drugs and how we can counteract the effects of antibiotic resistance.

Discovery of antibiotics

The potential of antibiotics was first demonstrated following the discovery and use of salvarsan, which was used in the first antibiotic drug trial of late stage syphilis patients and where impressive outcomes were observed [37]. Antibiotic discovery and use was further invigorated by the discovery of penicillin from a contaminated agar plate by Alexander Flemming [38]. Following the purification steps developed by Florey and Chain, the medicinal use of penicillin came into fruition[39]. The importance of this discovery was that microorganisms themselves had the ability to produce small chemical compounds that inhibited or killed other microorganisms. This led to the Golden era of antimicrobial discovery with most success being discovered by the Waksman antimicrobial discovery platform [40]. Following the boom in antibiotic discovery it was naively believed that chemistry would be able to produce novel antimicrobials. This approach generated fewer effective novel antimicrobials than expected at the time, but did generate better formulations, drug penetrations and drug delivery [41]. The impact of this created the antibiotic discovery deficit (Fig 2), and – coupled with the rise in antibiotic resistance – there is now a strong emphasis to discover novel antibiotics but also to further our understanding of our current antibiotics to optimize their use. The research involved in this PhD thesis of profiling drug resistance, in both the laboratory and from the clinical setting, aims at under-

standing bacterial antibiotic resistance evolution with the long-term goals of using antibiotics more effectively.

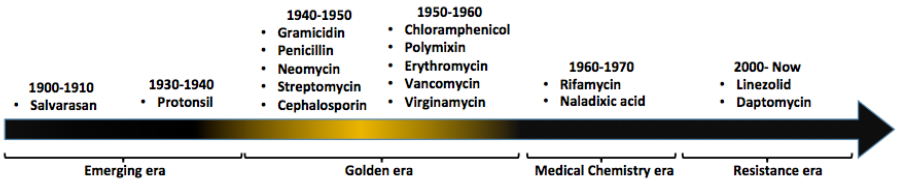


Fig 2. Time-line of antibiotic drug introduction date

Antibiotic Classes

To further understand our current antibiotic drugs it is important to know their chemical structure, as drugs that have the same structural class tend exhibit similar traits of effectiveness, toxicity and allergy potential [40]. Using structural chemistry to classify antibiotic drugs allows us to construct a generalized view of which antibiotic classes disrupt particular biological processes in the bacterial cell (Fig 3). Overall antibiotics disrupt three biological structures and synthesis processes essential for cell growth and maintenance. These are cell structure synthesis and maintenance, nucleic acid synthesis and protein synthesis. Further categorization can be done on the anitbiotic's other traits, such as their bacterial effect (i.e. bactericidal or bacteriostatic), the type of pathogens they target (i.e. Gram negative or positive or both), the spectrum of pathogens they target (i.e. narrow or broad-spectrum) and their pharmacological abilities. The classification and antibacterial traits of all antimicrobials are vital to know to be able to utilize these drugs to their maximum efficacy.

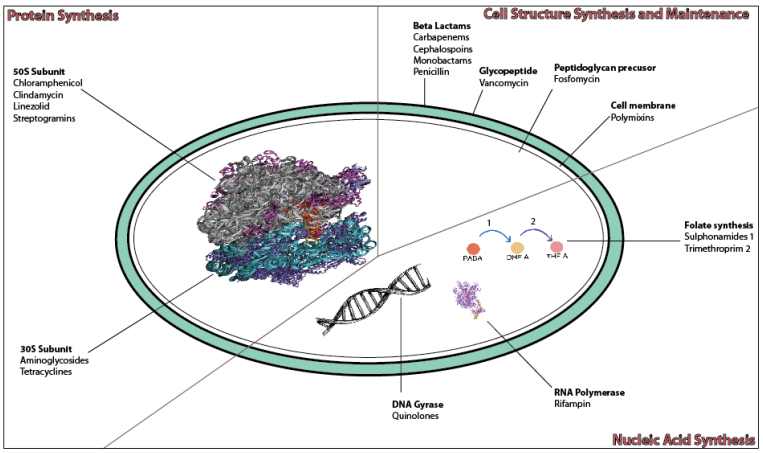


Fig 3. Generalized view of biological processes disrupted by different antibiotic classes. Top left are antibiotics that disrupt protein synthesis, top right is antibiotics that disrupt cell structure and maintenance and bottom right antibiotics that disrupt nucleic acid synthesis.

Antibiotic Resistance and Persistence

As previously mentioned bacteria have a tremendous capacity to evolve and overcome several survival limiting environmental factors. It is therefore no surprise that they also evolve to resist antibiotics. Antibiotic resistance from a bacteriologist perspective is the genetic adaptation of bacterial cells, which permits genetic altered cells to grow and divide in the presence of antibiotic concentrations that would normally kill or inhibit unaltered cells. Antibiotic resistance from a clinical perspective is related to the drug concentration tolerance and pharmacokinetics of the patient and the effects on the bacteria at this concentration [42,43].

Bacterial cells can alter their genome by acquiring plasmids (such as the CTX-M-15 plasmid disseminated in Enterobacteriaceae species [17]); by acquiring novel genomic material via viral vector (such as phages within the microbiome [44]) and by *de-novo* mutations (such as mutations observed in cystic fibrosis patients chronically infected with *P. aeruginosa* [45–47]). Despite the origins of the genomic antibiotic resistance adaptions, bacterial cells use four different types of antibiotic resistance mechanisms to evade drug effects. These mechanisms are: induction of multi-drug resistance (MDR) efflux pumps that expel the antibiotic drug from inside the cell; modification of the drug-target that prevent drug-target complexes forming; membrane modification that reduce permeability of the drug in to the cell; and production of enzymes that degrade the drug (Fig 4).

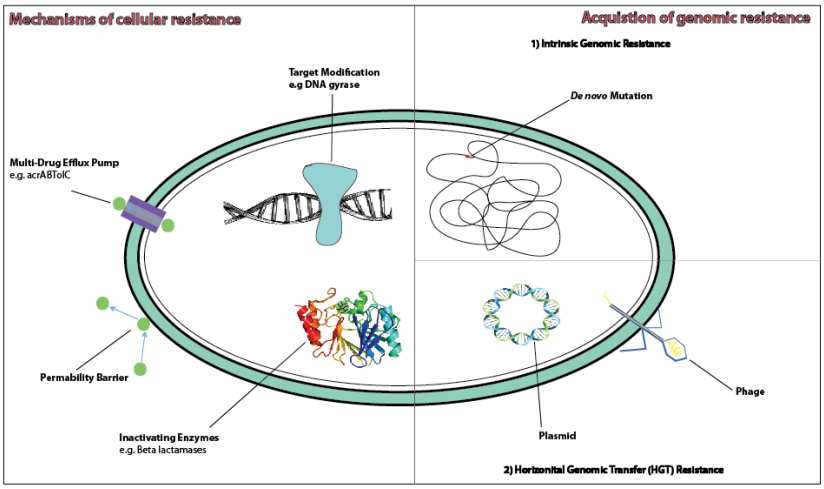


Fig 4. An overview mechanism of cellular resistance (on the left) and acquisition of antibiotic genomic resistance (on the right)

Examples of most of these molecular antibiotic resistance mechanisms are present within the two manuscripts presented at the end of this PhD thesis. For instance, in manuscript I there was induction of the acrAB-TolC efflux pump up-regulated in drug adapted bacterial populations by genomic modifications in the *acrR*, *marR*, *rob* and *soxR* genes. In manuscript II there was mutations that conferred up-regulation of the mexXY-oprM efflux pumps by genomic modifications in the *mexS*, *mexT* and *mexZ* genes. Whereas there was reduced permeability of drug in manuscript I, where mutations in the *cpxA* and *sbmA* genes confer AMK resistance. In both manuscripts it was shown mutations that confer molecular antibiotic resistance by drug-target modifications. In manuscript I these mutation were observed in *gyrA* and *fusA*, and in manuscript II these were *ftsI*, *gyrA*, *gyrB*, *parC*, *ftsI*, *rpoB*.

Usually most mutational antibiotic resistance variants come at a cellular fitness cost, due to the modification of essential cellular components. Therefore it has been assumed that if there was no antibiotic selection, antibiotic resistant variants would disappear [48]. Selection of resistant mutants can nonetheless occur at exceptionally low antibiotic concentrations, as demonstrated in the competition experiments performed by Gullberg et al. 2011. It is also important to state that some mutations to antibiotic agents come at little to no fitness cost and can easily be maintained within a population with no selection. Although most mutations do come at a fitness cost, these bacterial cells undergo further evolution to develop compensation mutations that restore fitness [49]. An example of this is demonstrated in Markusson et al 2009 work. Consequently, if sufficient time has allowed for bacteria to develop both antibiotic resistance mutations and compensation mutations, then simply eliminating the use of antibiotics will not rectify the situation. Hence, systematic approaches to understanding antibiotic resistance development

and methods to exploit it in antibiotic treatment are essential and will be discussed in the chapter application of profiling drug resistance.

From either a bacteriologist or clinical perspective, the best measurement of antibiotic resistance is using Minimal Inhibitory Concentration (MIC). MIC values by antimicrobial susceptible testing are required to deem if a bacterial species or sample is susceptible (S) (where treatment is likely to be successful), intermediate (I) (where its unknown if treatment will be successful) or resistant (R) (where treatment is unlikely to be successful to a given antibiotic) [50]. This will be covered in more detail in the next chapter on profiling drug resistance.

Impacts and dissemination of Antibiotic Resistance

The impacts of antibiotic resistance are considerable due to the fact that we are all possible stakeholders in requirement for antibacterial therapy (e.g. economically due to the cost of health care and for most medical treatment [51]). Many modern medical procedures rely on the use of antibiotics (e.g. prophylaxis and post-operative care for surgery [52], treatment for immunocomprised individuals [53] and treatment of sexually transmitted diseases [54]). There is a clear correlation that the more antibiotics we use the more bacterial antibiotic resistance occurs due to the large selective pressure we induce [55]. It is also important to state that there has always been a natural reservoir of antibiotic resistance that has existed before antibiotic usage. For instance, putative antibiotic resistance genes have been detected in ancient DNA studies (e.g. permafrost cores [56]) and the gut microbiome of 11th century pre-Columbian Andean mummy [57]). However, our misuse and over use will act as a strong selection to further disseminate antibiotic resistance and cause further antibiotic resistance evolution. This is especially so if the selection pressure is strong enough, such those encountered during human or animal antibiotic treatment [58], effluent from manufacturing plants [59] or wastewater treatment plants [60]. Therefore, to prevent or limit antibiotic resistance it is important for academics, clinical and industry to work together in developing new antimicrobials, optimizing treatment strategies and finding ways to minimize antibiotic usage to impede future antibiotic resistance.

Profiling and exploring drug treatment and resistance

Profiling drug resistance mainly relies on categorizing different isolates from different samples on their drug concentration abilities. Isolates can be collected from clinical samples, environmental samples and *in-vitro* adaptation experiments. The future of profiling drug resistance will also rely on molecular techniques on bacterial populations to provide a more comprehensive profiling of drug resistance. These methods will be elaborated on in this chapter.

Phenotypic quantification of antibiotic resistance

As mentioned in the previous chapter the best initial form of categorizing antibiotic resistance is by the drug's *in-vitro* MIC value, which allows the strain's antibiotic susceptible type be known. This can be measured either by broth dilution in incremental concentration steps or by epsilometer (E-test) assay. To perform these tests effectively, guidelines should be strictly adhered to [61]. This is to ensure the only variable of the assay is antibiotic drug concentration. Once a MIC value has been obtained following the clinical breakpoints on EUCAST website in accordance to the bacterial species [62], an antimicrobial susceptible type can be stated which can be S, I or R. The EUCAST clinical break-points have been determined by epidemiological studies of a large number of bacterial isolates and are regularly updated and can be viewed on method (MIC or disk diffusion), antimicrobial and species (EUCAST Antimicrobial wild type distribution of microorganism). However, these guidelines are often slightly relaxed in research facilities, where MIC is often translated into antibiotic inhibitory concentrations (IC) at different population levels such as IC90 and IC50 values [63–65].

Phenotypic quantification of two or more antibiotic drug agents can also be assessed. To evaluate this the fractional inhibitory concentration (FIC) can be measured following the checkerboard FIC methods [66], where agents are tested alone and together. From this an FIC index value can be calculated. Antibiotic combinational effects can be categorized as synergistic (more effective when used together), additive (the same effective when used to-

gether, equivalent to using a double dose) and antagonistic (less effective when used together) [67].

Further phenotypic evaluation of an adapting bacterial population can be performed by assessing the evolution of an antibiotic drug-pair compared to its single drug components. This is calculated by using the formula presented in Munck et al 2014 work, which relies on IC90 values of the drug-pairs and the single drug components before and after adaptation. From the calculated evolvability value, if a value ≥ 1 this implies that resistance to the drug-pair evolves to the same extent as the single drug components, whilst a evolvability value of ≤ 1 implies that resistance to the drug-pair evolves to the lesser extent than the single drug components. This calculation helps to provide information that can indicate drug cross-resistance or more importantly collateral sensitivity, a term that will be explained in the next chapter. For manuscript I the cryopreserved samples were obtain from Munck et al. 2014 work on phenotypic assessment on evolved bacterial populations drug-pair and single drug, where we further evaluated the populations by using molecular techniques.

Molecular quantification of antibiotic resistance

Molecular methods can reveal 'omics' changes (i.e. genomic, transcriptomic and proteomic changes) to bacterial isolates or populations depending on the procedure and method used. These methods can provide rapid and sensitive determination of antibiotic resistance from a wide range of samples.

The simplest method is PCR, where DNA from an isolate or a sample can be amplified with designed primers to see whether a resistance gene is present or not. An advance of this method is quantitative PCR (qPCR), where monitoring the amplification of the PCR product can verify the presence of given genes and their amount in the sample. The qPCR is seen as an attractive diagnostic tool as it is rapid, accurate and sensitive compared to cultivation based determination and can provide information in epidemiological route of transmission etc. PCR methods have been used to determine tetracycline resistance genes [68], methicillin resistance encoded *mecA* gene in Staphylococci and rifampicin resistance in *Mycobacterium tuberculosis* [69] and plasmid mediated ampC in Gram negative species [70]. However, there are two main limitations to these PCR approaches. The first limitation being that the presence of these antibiotic resistance genes does not mean that treatment of certain antibiotics will fail, due to the expression of these genes being low; and the second limitation being that novel molecular mechanism can easily be missed [69].

The first problem can be overcome by using transcriptomic or proteomic methods; where with transcriptomics direct gene expression can be analyzed, and with proteomics protein levels and modification can be evaluated. Overcoming the second problem requires a more in-depth analysis, such as using sanger sequencing and whole genome sequencing (WGS) on bacterial isolates to capture the novel mutations. This can easily be performed but is expensive. These sequencing methods are frequently used for clinical isolates, adaptation laboratory evolution studies, and environmental samples. Other approaches can be taken such as metagenomics where sequencing the DNA of the whole community can take occur. This allows for detection of the whole resistome, but is limited on the annotated antibiotic resistance genes in public gene databases [71]. Another limitation to this technique is that most metagenomic methods rely on NGS platforms that generate short sequencing reads which can make assembly of genomes into long contigs hard with limited mapping resolution. But unlike other methods it does allow for the whole uncultured community to be sequenced.

Therefore, our approach was in manuscript I to PCR amplify loci of interest in known antibiotic resistance genes and for manuscript II PCR amplify well known antibiotic resistance genes of interested and then use population sequencing on the MiSeq platform. We were fortunate that we only wanted to know the molecular resistance changes to our organism of interest that we could easily map to a reference. This allowed for us to generate amplicons to eliminate background noise, sequencing reads that were easy to map and directly detected variant and establish their frequency in the population.

Verification of molecular detection of antibiotic resistance

Unlike phenotypic assays, NGS data sets require validation of variants detected as these are mere observations. This can be done by two different approaches: using gene editing tools or literature mining to find validation results. In the first approach, modifications in an ancestral wild type are done by recombineering techniques such as homologous recombineering where SNPs and INDELS can be reintroduce [72], or by high-throughput SNP CRISPER-Cas9 technologies [73]. The second approach is the simpler of the two where validations have been previously been preformed, analyzed and values reported.

In manuscript I selected mutations were recombineered, and relative fitness to the ancestral wild type, IC90 to a given drug and persistence at different drug concentrations was reported. For manuscript II all treatment enriched

non-synonymous mutations were in the literature mined, those where recombineering and MIC difference were reported are complied in table 4.

Generation of clinical and environmental sample collections

Most bacterial collections comprise of isolates from samples where microbiology laboratories have processed the samples. Examples of these are *P. aeruginosa* isolate collection from sputum samples from cystic fibrosis patients [74,75] *Staphylococcus aureus* isolates from a teaching hospital [76]. However, especially in environmental samples, it is well known that only a small percentage of bacterial species can be isolated. Therefore many sample collections comprise of extracted DNA, which can later be analyzed by metagenomic techniques (e.g. pyrosequencing of antibiotic resistance-contaminated river sediments [77]). In manuscript II we used culture-independent methods to analyze our *P. aerugionsa* populations by extracting the DNA, amplifying our genes of interest and sequencing on the Miseq NGS platform. Validation of results was done with literature mining.

Generation of laboratory generated sample collection

To generate antibiotic resistance, sample collections tend to rely on adaptation evolution experiments to evolve isogenic naïve wild types to become drug resistant. Unlike clinical bacterial isolate, where evolutionary changes lead to antibiotic resistance, adaptation evolution experiments allow to regularly freeze samples to form cryopreserved stocks [78]. As the experiments are performed in controlled laboratory settings, scientific analysis of evolutionary phenomena are easier to analyze [79]. Therefore, in turn, replay experiments and tracking experiments can easily be performed.

Adaptation evolution experiments can be performed by two protocol approaches: a chemostat approach or a serial dilution protocol (Fig 5).

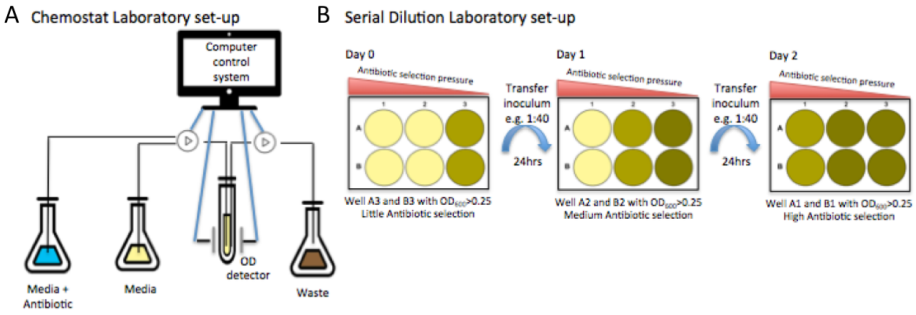


Fig 5. An overview of adaptive evolution protocols: (A) Chemostat laboratory set-up where a computer control optimizes the growth of the bacteria in the reaction vessel by continuously monitoring the OD and dynamically adjusting antibiotics concentrations; (B) Serial laboratory set-up where bacteria are grown in a microtire plate with increasing antibiotic concentration from left to right and bacterial replicated in different rows, by serial passaging bacteria can adapt to higher levels of antibiotic (right).

In the chemostat approach, bacterial populations are continuously exponentially grown by the constant nutrients given and waste and excess bacteria removed. An example is the development of the 'mobidostat' explained in Toprak et al 2013 work, where bacterial populations are constantly measured for optical density to measure growth rate and antibiotic drugs can be dynamically administered. This device was used to generate in parallel *E. coli* adapted populations to three drugs (chloramphenicol, doxycline and trimethroprim) for 20 days. From this study the analysis of evolutionary trajectories and end-point isolates were sequenced. Additionally, they also sequenced multiple time-points to understand the cumulative mutational development in the dihydrofolate reductase gene. A similar process which we used in the generation of manuscript I. The chemostat approach best simulates infections occurring within tissue or an organ where no transfer occurs [78]. The serial dilution approach of adapting bacterial populations grows bacteria for a given time period and then a small inoculum is transferred to a new vessel or well in increasing antibiotic concentration increments. An example is Munck et al. 2014 work, where in microtitre plates the wells that had the highest antibiotic concentration after incubation for 20 hours with an $OD_{600} > 0.25$ absorbance were re-inoculated at 1:40 dilution factor into fresh microtitre plates. This was done for 14 days. At the end of the experiment, bacterial populations from each drug condition and lineage had a representative isolate whole genome sequenced. This approach best simulates patient to patient transfer events [78].

Another alternative method for systematically examining antibiotic resistance is to use strain collections consisting of genetic mutants. Examples are the use of the KEIO strain collection where systematically studying the intrinsic resistome of *E. coli* to various drugs and examining the phenotypic response yielded important results [80,81]. This has also been applied to *P. aeruginosa* by using transposon generated libraries, which then can be tested in a similar manner to examine the intrinsic resistome[82–84].

In relation to the research work performed, in manuscript I genomic evaluation of the cryopreserved population samples was generated by serial dilution adaption evolution experiments. Whilst in manuscript II, if to be re-performed under strict laboratory conditions, a chemostat laboratory set up would probably be more applicable, due to the bacteria's permeant residence in the cystic fibrosis patient lung.

Applications of profiling drug treatment and resistance landscapes

The implication of profiling evolutionary landscapes in regards to antibiotic resistance are vital, for the ability to use antibiotic as an essential medical resource. Clinical antibiotic resistance is unfortunately not hard for bacteria to achieve due to the micro-niches in the body e.g. in the lungs, epidermis and joints [85]. Especially in these micro-niches bacteria experience sub-inhibitory antibiotic concentrations, which impede but not prevent growth [85,86] and even allow evolution to occur to circumvent any negative effects. Therefore, through this next chapter I will discuss the application of profiling drug resistance landscapes.

Traditional approaches to applying drug profiling for treatment, with limited antibiotic resistance outcomes

Within microbiology it has been naively accepted that the development of bacterial antibiotic resistance can be impeded by the use of drug combinations, especially those that exhibit synergistic effects. The assumption was that bacteria developing resistance to one drug was more likely than developing resistance to two drugs simultaneously [87]. Traditionally in the clinical setting most antibiotic drug combinations are chosen due to their different cellular targets [88], but despite many antibiotics attacking different cellular targets bacteria often evolve the same mechanisms to resistant antibiotics. These mechanisms are commonly known as cross-resistance mechanisms (e.g. are the multi-drug resistant efflux pumps and modifications to pleiotropic genes [89]). However, there are exceptions; e.g. it has been frequently found by *in-vitro* assays that many classes of antibiotics have synergy with aminoglycosides [90–92]. This did have great clinical success and now with understanding the molecular mechanisms we can elucidate ways to utilize antibiotics more effectively. In the next sub-chapter, I will further explain the mechanisms of why aminoglycosides exhibit synergistic qualities with other antibiotic drug classes.

Novel methods to systematically review drug reactions

Traditional microbiology could only assess antibiotic resistance by phenotypic assays or by low throughput sanger sequencing to reveal genomic changes. With developments in molecular and automation technologies it is easier to study phenotypic drug responses and study cellular molecular changes. These new methods have been able to resurrect old hypothesis, such as collateral sensitivity, test them and provide important information to ascertain the application validity.

Systematic studies can explore a bacterial species' reaction to large volume of antibiotics. This can be done by investigating bacterial collections of gene knockouts, transposon mutant libraries, adaptation populations and endpoint isolates, and clinical or environmental isolate collections. Most studies have focused on WGS and reported where genomic resistance has occurred. This could provide critical intrinsic antibiotic resistance information (e.g. the dihydrofolate reductase gene in trimethoprim resistance [93]). Other studies have lead in promoting antibiotic treatment ideas and strategies, which hopefully can be used in clinical application once certain parameters have been satisfied.

Ideas promoting novel targets were generated following the development of the Keio collection of approximately 4000 single gene knockouts. This allowed scientists to test the Keio collection with different antibiotics. The first study by Tamae et al 2008 showed that with high-throughput screening of 7 drugs, there were genes that made a given bacterial strain hypersensitivity to a given drug. This was further extended in Lui et al 2010 work with 22 additional antibiotics. Both studies showed the complexity of the intrinsic resistome, and especially by targeting pleiotropic genes by co-drugs or antibiotic adjuvants, there was renewed hope in antibiotic treatments. Transposon libraries in *P. aeruginosa* also have shown promise in exploiting intrinsic resistome weak-points that could be useful for co-drugs or antibiotic adjuvants [82–84]. This produced the revival of an old idea of collateral sensitivity [94]. In this work bacteria that were made resistant to a given antibiotic agent became more sensitive to another. This phenomenon was the main focus in Imamovic and Sommer 2013 work. By adapting *E. coli* bacterial populations to 23 antibiotics and evaluating drug susceptibility profiles, collateral sensitivity and cross-resistance drug networks were established. After finding drug pairs that exhibited collateral sensitivity, a demonstration of drug cycling with gentamycin and cefuroxime was shown to be successful. Adaptation to each of the drugs was done sequentially and MICs of both drugs performed; the drug that was used for adaptation showed increased resistance whilst the other drug had a severely decreased MIC value. The use of drug networks displaying collateral sensitivity has also been explored by

Lazar et al. 2014, combining *in-vitro* adaptation of *E. coli* and whole genome sequencing to 12 antibiotic drugs with 5-6 replicates. It was later elucidated that aminoglycoside mutants often change key components required for energy production and permeability, whereas other resistance mutants e.g. chloramphenicol mutants require a higher demand of energy, e.g. mutants that up-regulate MDR efflux pumps. Using a similar approach, in Munck et al. 2014 work the experiment was performed on 10 drug-pairs and 5 single drugs done in triplicate for 14 days, showing that the drug pairs which involve amikacin and another antibiotic agent such as chloramphenicol can limit the development of antibiotic resistance.

To further elucidate these findings within the adaptive bacterial populations I used amplicon deep sequencing of around Munck et al 2014 end-point mutations. From this we discovered that the collateral sensitive drug pair AMK-CHL still developed resistance mutations in acrAB-TolC multi-drug resistant efflux transcriptional repressor genes, but did not develop mutations in the *fusA* and *sbmA* genes that is typical for AMK resistance development. These studies reported the genomic trade-offs that bacteria must make to become resistant to a given antibiotic drug, but this could leave them more vulnerable to another type of antibiotic drug. Therefore, using collateral sensitive drug cycling networks and collateral sensitive pairs tools could be effective to increase the longevity of antibiotic therapy until new agents are available [95].

Transfer *in-vitro* drug profiling results into possible clinical application

To transfer these *in-vitro* findings from the laboratory to clinical practice, an expansion of data must occur regarding the use of different clinically relevant pathogens and isolates [95]. Another consideration is antibiotic's pharmacodynamics parameters (that describe the impact of an antimicrobial agent on the target organism) and the pharmacokinetic parameters (that describe the availability of an antimicrobial agent in the target organ or tissue), which will provide information regarding the plausibility of using these agents within the patients [43]. It would be of interest to look into the long term effects of using antibiotic collateral sensitive treatment as a tool for possibly treating chronic infections, such as chronic pseudomonas infections. However today little is known of the bacterial population genomic divergence of antibiotic resistant variants especially in individuals inflicted with chronic infections. In manuscript II we begin to look into this by studying 19 genes in cystic fibrosis patients that have chronic p. aeruginosa lung infections whilst undergoing hospitalized treatment. We could see a vast array of

genomic mutant sub-populations present in the sputum samples. Therefore, understanding bacterial population divergence, sub-populations and the uses of different antibiotic and their deployment could help towards the treatment of bacterial pathogens.

Chapter 5. Concluding remarks and future perspectives

As stated through out this PhD thesis, bacteria have many advantageous traits that allow them to evolve to a vast array of environmental conditions. These traits are their: short generation time, large population capabilities and their rate of spontaneous mutations. By following adaptive evolution of bacteria, we can observe evolution in real time [96] and freeze regular samples for later tracking genomic events as seen with in the two manuscripts of this PhD.

The bacterial evolution studies within this PhD thesis are all related to intrinsic antibiotic resistance, within bacterial populations. Our molecular pipelines could detect SNP and INDEL variants but not clearly detect gene duplication events.

In manuscript I, deep amplicon sequencing of antibiotic loci were tracked over time to establish genomic events occurring in adapted collateral sensitive drug-pair bacterial populations, in adapted collateral resistant drug-pair bacterial populations and in adapted single drug bacterial populations. Our finding from this research indicated that adapted collateral sensitive drug-pair populations e.g. populations adapted to AMK-CHL stopped developing *fusA* and *sbmA* mutants but continue to develop mutants in other gene loci. In the adapted collateral resistance drug-pair these finding did not occur. Consequently, by using antibiotic collaterally sensitive drug pairs, evolution of antibiotic resistance can be limited but not completely inhibited it. We also observe how mutational variants appear in the population and how their dynamics follow the rules describe by others such as clonal interference, population fixation, and periodic selection occur [34,35,97,98].

Whilst in manuscript II, 19 *P. aerugiosa* antibiotic resistance and pathogadaptive whole genes were sequenced and analyzed from DNA extracted from chronically infected cystic fibrosis patients undergoing hospitalized antimicrobial therapy. The results of this manuscript indicated that there was a vast array of genomic variants produced during antimicrobial treatment; most of these were synonymous mutations. Therefore, we thought there could be environmental factors such as quorum sensing mechanisms endog-

enous to *P. aerugiosa* [99] inducing genomic divergence or the use of the antibiotics themselves that could be inducing genomic divergence by mechanisms such as SOS response[100]. We then examined the non-synonomous mutations within the population and observed effects of clonal interference that has been well documented through literature by adaptation experiments in other Gram negative species [98,101]. These events are probably important for the development of antibiotic resistance within the bacterial populations to find mutations are effective against antimicrobials that compromise fitness the least. Most of our novel enriched variants were not described within the current scientific literature, with the exception of 11 variants, where three had follow up studies where the MIC increase reported.

These studies have generated important but limited information. As mentioned by Hancock 2014, an expansion in antibiotic resistance information is required in different bacterial species, to several antibiotics and treatments, from different bacterial population locations.

From the work here I hope I can expand in the future by developing larger data-sets regarding antibiotic resistance within bacterial populations, by analyzing data from both systematic *in-vitro* experiments and clinical samples. As well as developing sequencing pipelines to capture genomic events across the whole genome within bacterial species populations, so duplication events could also be captured.

Maybe it is an ambitious statement to finish on, but by understanding factors that induce genomic variation we can hopefully find the mechanisms to impede it, such as the quasi-Lamarckian mechanisms that induce neo-Darwinism evolution in antibiotic resistance. Then using co-drug or antibiotic adjuvants [81,84,102,103] that target these quasi-Lamarckian mechanisms, suppression of antibiotic resistance development can ensure the longevity of modern medical treatments.

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Manuscripts

Manuscript I: Time-resolved tracking of mutations reveals strong clonal interference during antimicrobial adaptive of *Escherichia coli* to single and drug pairs.

Time-resolved tracking of mutations reveals strong clonal interference during Escherichia coli antimicrobial adaptive evolution to single and drug-pairs.

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Abstract

Understanding the evolutionary processes leading to antibiotic resistance can help to achieve better treatment strategies. Genomic characterization of antimicrobial adaptation evolution experiments is commonly done by sequencing representative isolates from end-point adapted populations. Consequently, little is known about the dynamics of the resistance alleles during adaptation. Here we use population sequencing to monitor genetic changes in key adaptive loci at several time-points during an adaptive evolution experiment to five different antibiotic conditions. We monitor the mutational spectrum in putative resistance alleles in lineages evolved to single antibiotics (amikacin, chloramphenicol and ciprofloxacin) as well as antibiotic combinations (amikacin + chloramphenicol and chloramphenicol + ciprofloxacin). We find that lineages evolved to antibiotic combinations exhibit

different dynamic resistance adaptation profiles compared to single drug evolved lineages. Analysis of allele frequency dynamics indicates interactions between specific mutations. We termed these clonal interference allelic interactions, super-imposing allelic interactions and population allelic fixation events. To assess our observations a sub-set of mutations were introduced into the ancestral wild type. The resulting stains were assessed on relative fitness, IC90 fold change from the ancestral wild type and mutant allelic persistence abilities.

Keywords: Antibiotics, allelic dynamics, population frequency sequencing

Introduction

Bacteria have an impressive ability to adapt to changes in their environment. Their large population sizes and short generation times allow them to rapidly evolve and adapt in response to environmental perturbations. In addition, their ability to horizontally acquire new genetic material enables them to quickly expand their functional capabilities. Consequently, bacteria have evolved to overcome stress from a wide range of xenobiotic including disinfectants (Gerba, 2015), metal ions (Sütterlin et al., 2014) and antibiotics (Dantas and Sommer, 2014; Gullberg et al., 2011; Piddock, 2012; Sommer et al., 2010).

In the case of more severe perturbations such as high-level antibiotic exposure, or other xenobiotic perturbations the regulatory plasticity is not sufficient and the bacteria have to evolve new traits to survive (Lindsey et al., 2013). This either occurs via horizontal acquisition of new genes or through mutations in existing genes. In the latter case, random single nucleotide variants (SNVs) or insertions/deletions (INDELs) in the genome drive the evolution of new traits. The ability of a mutated allele to establish within a population depends on the complex interplay between the positive selection for the conferred benefit of the mutation and negative selection against any associated fitness cost of the mutation. By identifying SNVs and INDELs at the population level at different time points during adaptation to a physical or chemical perturbation, it is possible to uncover the trajectories through which a population evolves. Studies of such evolutionary

61 trajectories have identified the mutational landscape that populations undergo
62 when adapting to different diverse perturbations such as; glucose as a limiting
63 nutrient (Barrick et al., 2009; LaCroix et al., 2015) and antibiotic exposure (Feng et
64 al., 2016; Toprak et al., 2011; Zhang et al., 2015).

65 An interesting phenomenon that often accompanies the mutational responses to an
66 environmental perturbation is the emergence of collateral effects. This
67 phenomenon describes the situation where the adaptive mutations acquired in
68 response to one perturbation affect the cell's tolerance to other perturbations. Such
69 collateral effects have been found in bacteria (Imamovic and Sommer, 2013; Lázár
70 et al., 2013; Munck et al., 2014; Oz et al., 2014; Pál et al., 2015; Szybalski and
71 Bryson, 1952), viruses (Miedema et al., 2013) and human cell lines (Cerezo et al.,
72 2015; Zhao et al., 2016).

73 Fundamentally, collateral changes can be divided into two categories; collateral
74 resistance and collateral sensitivity. Collateral resistance is the situation where
75 increased tolerance to one xenobiotic perturbation also gives increased tolerance
76 to other xenobiotic perturbations. In contrast, collateral sensitivity describes the
77 situation where increased tolerance to one perturbation is accompanied by
78 increased sensitivity to other perturbations. In a recent study we investigated how
79 bacterial populations respond to a dual selection pressure relative to a single
80 selection pressure (Munck et al., 2014). Over the course of fourteen days bacterial
81 populations were adaptively evolved to either single antibiotics or combinations of

two antibiotics. Interestingly, we found that the presence of collateral sensitivity between two drugs significantly limited the populations' capacity to evolve resistance. In the current study we follow the evolutionary events at key genetic loci in bacterial populations that have been exposed to increasing concentrations of either a single antibiotic or combinations of two antibiotics (Munck et al., 2014). We investigated lineages evolved to amikacin [AMK], chloramphenicol [CHL], ciprofloxacin [CIP], amikacin + chloramphenicol [AMK-CHL] and chloramphenicol + ciprofloxacin [CHL-CIP]. The AMK-CHL drug combination was selected since it was found to generate evolutionary tension suppressing evolution of antimicrobial resistance, whereas the CHL-CIP combination was chosen since the component drugs have complementary evolutionary trajectories that enhance resistance evolution towards the combination (**Figure 1A**). In this study amplicon population sequencing and genome engineering were used to characterize the evolutionary dynamics in response to the individual antibiotics and antibiotic combinations (**Figure 1B**).

Materials and Methods

Bacterial Strains

The bacterial strain used in this study was MG1655, an *E. coli* K-12 MG1655 wild-type (Blattner et al. 1997) for the adaptation evolution experiment and generation of sample collection. Further characterization experiments were done on successfully recombined strains to reintroduce given mutations, the strains were: *E. coli* K-12 MG1655:*marR* C328T, *E. coli* K-12 MG1655:*rob* G467A, *E. coli* K-12 MG1655:*marR* C328T + *rob* G467A, *E. coli* K-12 MG1655:*gyrA* A260G, *E. coli* K-12 MG1655: *marR* T251A, *E. coli* K-12MG1655:*gyrA* A260G + *marR* T251A, *E. coli* K-12MG1655:*soxR* 378_380delGCG and *E. coli* K-12MG1655: *gyrA* A260G + *soxR* 378_380delGCG.

Sample revival and DNA extraction

Saved microtitre plates from Munck et al 2014 were defrosted for 30 min at 4°C, to prevent aerosol contamination plates were centrifuged at 300g for 5 sec before seal removal. To revive our samples, 150µL samples were transferred to a new tube with 500µL LB media and incubated at 37°C for three hours. Genomic DNA was extracted by using Genomic Mini kit in accordance to the protocol (A+A biotechnologies).

Resistance allele Primer design

The loci selected for sequencing were chosen based on knowledge of their involvement in antibiotic resistance and the mutation profile in the end-point sequenced original evolved lineages and population level loci sequencing was

performed on day 2, 4, 6, 8, 10 and 10 (**Figure 1A**). For this study we monitored mutations for AMK: *cpxA*, *fusA* and *sbmA*; for CHL: *acrR*, *marR*, *rob* and *soxR*; for CIP *acrR*, *gyrA* and *soxR*; for AMK + CHL: *acrR*, *cpxA*, *fusA*, *marR*, *rob* and *soxR*; for CHL + CIP: *acrR*, *gyrA*, *marR*, *rob*, *soxR*. We believe these loci cover important genomic locations to begin to unravel important interaction in intrinsic antibiotic resistance. Primers were designed with overhangs to allow barcoding with Nextera XT indices in accordance to Illumina 16S Metagenomic Sequencing Protocol (Illumina Protocol Online) to amplify 12 different genomic regions (**Supplemental Table 1**) in known where resistance causing SNPs. All primers were tested on *E. coli*-K12 wild type genomic DNA and PCR products were verified by 1% agarose gel electrophoresis with GelRed (Biotium).

Amplicon library preparation and sequencing

All samples were amplified using the primers in (**Supplemental table 1**) and following the description in the above section for each drug condition. Each PCR reaction was done in 0.2mL sterile PCR tubes and consists of 10 μ L phusion flash high-hidelity PCR master mix (ThermoFischer Scientific), 0.5 μ M forward primer, 0.5 μ M reverse primer, 1 μ L DNA template and H_20 to a total reaction volume of 20 μ L. The PCR amplification used an initial denaturation of for 98^{o}C 30 s, 30 cycles consisting of a 98^{o}C for 10 s denaturation, 65^{o}C for 10 s annealing, 72^{o}C for 15 s elongation, 72^{o}C final extension for 60 sec then hold at 4^{o}C. To validate correct

amplification gel electrophoresis was performed with 1% agarose gel and stained with ethidium bromide. After verification all the different loci amplicons that belonged to the same drug-condition, lineage and time-point were pooled together. PCR products were cleaned by Agencourt AMPure XP PCR purification system used according to their online protocol (Beckman Coulter 2013). The barcoding of the amplicons was done by a second PCR amplification and was done in 0.2mL sterile PCR tubes and consists of 25 μ L Phusion Flash High-Fiedelity PCR master mix (ThermoFischer Scientific), 5 μ L Nextera XT index 1 (N7XX), 5 μ M Nextera XT index 2 (S5XX) (Illumina), 5 μ L pooled amplicons and H_20 to a total reaction volume of 50 μ L. The PCR amplification used an initial denaturation of for 98^{o}C 30 s, 8 cycles consisting of a 98^{o}C for 10 s denaturation, 65^{o}C for 10 s annealing, 72^{o}C for 15 s elongation, then 72^{o}C final extension for 60 sec then hold at 4^{o}C. Barcoded amplicons were cleaned according to Agencourt AMPure XP PCR purification systems (Beckman Coulter 2013). Barcoded amplicons were measured for DNA concentration with Qubit dsDNA HS Assay kits and measured on Qubit Fluorometer (Thermo Fischer Scientific Inc 2015) and average fragment size with Agilent DNA 1000 kit and measured on Agilent 2100 Bioanalyzer instrument (Agilent Technologies 2013). Then barcoded amplicons were pooled from fragment size and DNA concentration values to form a sequencing library based. The sequencing library was sequenced on the MiSeq sequencing platform (Illumina).

Sequence Analysis

All raw sequencing read from our amplicon sequencing have been deposited in the Sequence Read Archive (SRA) under bioproject accession PRJNA328094. Sequencing reads were mapped to the reference genome (GenBank accession NC_000913) using CLC genomics workbench (Qiagen). These files were exported and used for coverage analysis. To verify sequencing coverage mapping read files from CLC genomic workbench and using the statistical program R (R Core Team, 2014a). Coverage plots were generated and any amplicons than were below our set threshold of 1000 read coverage were re-done until our threshold was achieved, to ensure good quality data (**Supplemental Figure 1-5**). To detect variants, all mappings were analyzed with the basic variant detection calling function in CLC genomic workbench (Qiagen). Files were further analyzed using the program R (R Core Team, 2014b), our detected variants are listed in **Supplemental Table 2**.

Recombineering for direct mutagenesis

For single mutant variants, the ancestral wild-type *E. coli* K-12 MG1655 was transformed with the transient mutator plasmid PMA7sacB (GenBank accession KT285941)(Lennen et al., 2016). For each single variant mutations two recombineering cycling rounds were done with *E. coli* K-12 MG1655 harboring the PMA7sacB plasmid this was in accordance to the MAGE cycling with the single-stranded oligonucleotides (ss-oligo) with verification primers (**Supplemental**

Table 3) and plasmid curing described by Lehnen et al. 2016. Direct mutagenesis verification was confirmed by Sanger sequencing, done with Eurofins Mix2Seq kit and following their instructions (Eurofins Genomics). After verification, double mutant variants were created by taking one single mutant variants from the observed double-mutant pair and transforming with the with the PMA7sacB plasmid, recombineering with the other mutant variant ss-oligo was done in two rounds as previously described and plasmid curing was also performed.

Characterisation of recombineered mutation variant strains

Relative growth rate as indictor of fitness was done for all recombineered strains and wild type. To measure relative growth fitness, overnight cultures in LB medium were diluted to 1 x 10^6 CFU/mL, then added to 96-well microtitre plate with a medium negative control all performed with 8 biological replicates, with two technical replicates to allow calculation of the relative growth rate. Growth of the samples was done at 37^{o}C with shaking for 10s and kinetic measurements on ELx808 absorbance microplate reader with Gen5 software (BioTek Instruments Inc). Optical density measurements were taken at 630 nm (OD_{630}) every 10 min. Exponential growth was defined as OD_{630} values between 0.02 and 0.1. To calculate relative growth R statistical software was used to find the doubling time (Td) of each of the strains and the standard deviation (Td SD), then to calculate relative growth rate, the recombineered strain Td value was divided by the wild type Td value. Selective advantage was measured by performing IC90 determination assays

to the antibiotic drug condition where the mutations were observed. IC90 determination was performed in 96-well microtitre plates with 2-fold drug gradient consisting of 10 drug concentrations prepared in LB broth, each well had a volume 100 μ L and was inoculated with 1 μ L bacterial culture to provide well start inoculum 1x10 CFU/mL, each plate had 8 biological replicates. Plates were incubated for 18h at 37^{o}C, and OD600 was read on Synergy H1 plate reader (BioTek Instruments Inc).

Results

Selection of alleles for population sequencing.

We specifically targeted putative resistance alleles identified by Munck et al. 2014 through whole genome sequencing of evolved isolates. As template we used DNA isolated from evolving populations sampled every 48 hours of the experiment. The following genomic loci (and drug conditions) were studied: *cpxA*, *fusA* and *sbmA* (AMK); *marR* and *rob* (CHL); *acrR*, *gyrA* and *soxR* (CIP); *cpxA* and *marR* (AMK + CHL); *acrR*, *marR*, *gyrA* and *soxR* (CHL + CIP). These genes are implicated in either multi-drug resistance (collateral resistance) or specific-drug resistance (collateral sensitivity). The genes implicated in multi-drug resistance include global regulators that induce up-regulation of acrAB-TolC multi-drug efflux systems, i.e. *acrR*, *marR*, rob and *soxR* (Chubiz et al., 2012a). Mutations in these global regulators often have generalized cross resistance effects to many antibiotic

classes. Consequently, we termed these as mutations in cross resistance gene loci. In contrast, genes implicated in specific-drug resistance are *gyrA* mutations (Fu et al., 2013), that confer CIP resistance, and *cpxA* (Girgis et al., 2009), *fusA* (Johanson and Hughes, 1994)and *sbmA* (Chubiz et al., 2012b; Puckett et al., 2012), that confer AMK resistance.

Mutant allele dynamics differ between single drug and drug combination evolved populations

Our sample collection consisted of 90 samples from 15 bacterial populations. These were triplicates of adaptive evolution experiments to 5 drug conditions. 3 of these were single drugs: AMK, CHL, CIP; and 2 were drug combinations: AMK-CIP and CHL-CIP. The triplicate parallel lineages were assigned letters from A-C and were sampled on 6 time-points (Day: 2, 4, 6, 8, 10 and 12) (**Figure 1A**). From our sample collection, we extracted genomic DNA, amplified loci of interest, verified products by gel electrophoresis, pooled together amplicons comprising targeted alleles based on drug-condition and time-point, barcoded the amplicons with indices in a second PCR step, and pooled them together to form a sequencing library. The library was then sequenced using the MiSeq sequencing platform to establish mutational population frequency dynamics in antibiotic resistance conferring loci (Material and Methods and **Figure 1B**). A minimum coverage of 1000 sequencing reads per amplicon was required before further data processing (**Supplemental Figure 1-5**).

From our results we wanted to compare the allele frequency dynamics of single drug evolved populations versus combination drug evolved populations. We observed differences in the cumulative frequencies of mutations of some genes in the combination condition compared to their single drug components (**Figure 2A).** There were minor differences in gene loci mutational cumulative frequencies, except for the collateral sensitive gene loci of AMK-CHL compared to AMK. It is assumed, that antibiotic combinations would suppress antibiotic resistance evolution compared to drug monotherapy. Yet, we have previously shown that this is not always the case; instead only combinations with collateral sensitivity interactions seem to suppress drug evolution (Munck et al., 2014). We did a Holm-Sidak multi-comparison two-way ANOVA statistical test to compare the cumulative mutational frequencies between different drug conditions and sequenced gene loci characterized in this study. We found spurious differences between single and combination drug conditions in their respective cumulative mutational frequencies. However, two gene loci showed consecutive significant differences over two or more time-points. These were the *fusA* gene and *sbmA* gene, where mutations were significantly less frequent in AMK-CHL drug evolved populations compared to the AMK drug evolved populations (p-value ≤0.001, Holm-Sidak multi-comparison two-way ANOVA test) (**Figure 2B**). This was also observed in the work of Munck et al 2014 and likely results from collateral sensitivity to CHL caused by these mutations (de Cristobal et al., 2008; Macvanin et al., 2005).

Populations exhibit complex dynamics of mutant alleles during antibiotic resistance evolution

To elucidate important mutant alleles within our populations, we selected all alleles that appeared in at least one time-point with a population frequency ≥30% (Supplemental **Figure 6**). We observed 5 mutations that occurred in parallel evolution events in different adapted populations (AMK: *cpxA* Trp184Arg, *fusA* Pro610Gln; AMK-CHL: *acrR* Lys55Glu; CIP and CHL-CIP: *gyrA* Ser83del; CHL-CIP: *soxR* Arg20Cys). Out of these mutations, 2 have not previously been reported in the literature, namely *acrR* Lys55Glu in AMK-CHL populations and *cpxA* Trp184Arg in AMK populations. We speculate that the *acrR* Lys55Glu mutation confers resistance to the CHL component in the AMK-CHL populations by up-regulation of the acrAB-TolC multi-drug efflux pump whereas *cpxA* Trp184Arg confers AMK resistance by modifying membrane stress response.

The most prevalent mutation in the evolved bacterial populations was *gyrA* Ser83del, as it was observed in 3 CHL-CIP and 2 CIP populations at several time-points. The *gyrA* gene often mutates at amino acid position 83 however it has only been reported once that the mutation was due to a deletion of the codon (Jaktaji and Mohiti), as seen within our data. We saw this mutation in high prevalence, especially in the CHL-CIP lineage A, where on day 12 it was observed at a frequency of 100% in the population. However, this mutant allele was not detected in Munck

et al 2014 work possibly due to the use of whole genome sequencing of a representative isolate from the population.

Within our data we observed three distinct accumulation patterns of different mutant alleles (**Figure 3A**). We termed these: discordant allele frequency dynamics, where the mutant alleles have opposing frequency trajectories to each other (**Figure 3A (i-iii)**); super-imposing allele frequency dynamics, where the mutant alleles have the same frequency trajectories (**Figure 3B (i-iii)**); and accumulating allele frequency dynamics, where one allele accumulates in the population following the accumulation of another allele (**Figure 3C (i-iii)**).

We observed discordant allele frequency dynamics in three populations: CIP lineage B, AMK lineage B and CHL Lineage C. For CIP lineage B we were able to infer direct antagonistic interactions as the mutations were within the distance of a single read. We observed that the individual reads only had an amino acid change in *gyrA* at position Ser83del or Asp87Gly for the time-points 4 to12. These interference patterns could be a result of mutational incompatibility or clonal interference, but further experimentation is required to deduce which effect is responsible for our observations.

We observed super-imposing allele frequency dynamics in three populations: AMK Lineage C, CHL-CIP lineage A and CIP lineage A. For all three populations the two mutant alleles have similar population frequency over multiple-time points

(**Figure 3 (C) (i-iii)**). Such accumulation patterns likely result from the quick succession of two separate mutations within a cell. Since, our population sequencing was performed only every 48-hours we have likely not been able to resolve occurrence of such successive events.

For the accumulating allele frequency dynamics we observed one allele that occurred at high frequency in the population followed by the accumulation of a subsequent allele. This was observed in three populations: AMK lineage B, CHL Lineage B and CIP Lineage A. These observations could be the result of two possible effects, firstly the hitchhiking effect where a neutral mutant allele is co-occurs with a beneficial mutant allele due to the selective advantage provided by the beneficial allele the neutral mutation is therefore irreversible (Taddei et al., 1997; Tenaillon et al., 1999) or secondly through positive or neutral epistasis (Fogle et al., 2008; Sniegowski et al., 1997). Unlike the super-imposing trajectories, the original mutation can be readily identified.

Recombineering permits validation of allelic interactions

To follow up on our observations we used recombineering to engineer strains with our observed mutant alleles. This was done with a sub-set of detected mutations exhibiting super-imposing or discordant accumulation patterns. By measuring the relative growth rate and antibiotic tolerance of the isogenic mutants compared to the ancestral wild type we were able to deduce why some mutation interactions occurred (**Figure 4A (i-ii)**).

For our discordant mutational allelic frequency dynamic pair observed in CHL lineage C population we observed that the *rob* G467A allele became dominant in the population and *marR* C328T was lost from the population from day 6 onward (**Figure 3A (iii)**). Therefore we recombineered 3 bacterial strains MG1655:*rob* G467A, MG1655: *marR* C328T and MG1655: *marR* C328T+*rob* and tested each strain for relative growth rate and CHL IC90 fold increase from wild type (**Figure 4A (i)**). From our recombineering results we deduced that the MG1655:*rob* G467A strain was selected due to its selective advantage to the antibiotic perturbation in comparison to MG1655: *marR* C328T strain (**Figure 4A (i)**). As the recombineered strain MG1655:*rob* G467A had IC90 fold increase of 6 from the ancestral wild type compared to MG1655: *marR* C328T strain with a fold increase of 5.5. Accordingly, we hypothesize that the observed accumulation patterns in the CHL lineage C population result from clonal interference and not mutational incompatibility. To validate this assumption we recombineered the strain MG1655: *marR* C328T+*rob* G467A, this strain was viable and also had a strong CHL tolerance as it had an IC90 fold increase of 11 compared to the ancestral wild type. However, the strain did have a reduced relative growth rate, in comparison to the single mutate allele components and in the ancestral wild type.

For our super-imposing mutations we tested our observation in the CHL-CIP lineage A population, where *gyrA* 247_249delTCG and *marR* T251A appeared at the same frequency from day 8 (**Figure 3B (ii)**). Therefore we recombineered 3

bacterial strains MG1655:*gyrA* 247_249delTCG MG1655: *marR* T251A and MG1655: *gyrA* 247_249delTCG+ *marR* T251A and tested each engineered strain for relative growth rate and CHL-CIP tolerance compared to the ancestral wild type (**Figure 4A (ii)**).

We find that the bacterial cells possessing both mutant alleles *gyrA* 247_249delTCG and *marR* T251A have a strong selective advantage, as the IC90 of the double mutant was 11-fold higher than the ancestral wild type, compared to 4.3-fold and 6.6-fold for the single mutants *gyrA* 247_249delTCG and *marR* T251A, respectively. The double mutant did have a reduced relative growth rate of 0.75 when grown in antibiotic free conditions. We believe these mutations complement each other: *gyrA* 247_249delTCG confers direct resistance against CIP (Fu et al., 2013) while *marR* T251A induces up-regulation of the acrAB-TolC multi-drug efflux pump causing collateral resistance to both CHL and CIP (Chubiz et al., 2012a).

Our previous observations coupled with our recombineering results show that bacteria sample many mutant allele types. Mutant alleles that are disadvantageous dissipate quickly from the bacterial population such as the three mutant allelic losers in discordant mutation allelic dynamics (CIP Lineage B: *gyrA* Ser83del; AMK Lineage B: *cpxA* Trp184Arg; CHL Lineage C: marR Gln110*). Whilst those that aid survival persist such as the interactions demonstrated in the super-imposing or accumulating mutational allelic dynamics (AMK Lineage B: *cpxA* Asp31Tyr + *fusA* Pro610Gln; CHL Lineage B: *marR* Arg86Trp + *rob* Arg150His; CIP Lineage A: *gyrA*

Asp 87Tyr + *gyrA* Gln75Ser; CHL-CIP Lineage B: *gyrA* Ser83del + *marR* Val 84Glu). These mutant alleles will either become part of the population's adaptation history or will be eventually lost to a new mutant allele successor.

Discussion

From previous work by Munck et al 2014 we observed mutations that counteract the effects of antibiotics both by collateral resistance and collateral sensitive mechanisms. The Munck et al 2014 study, as well as many others relies on end-point genome sequencing of representative isolates to investigate evolved populations. In this study we show the importance of using next generation re-sequencing tools to further evaluate adaptation evolution in mutated loci over time at the population level. Using this approach, we find that selecting drug-pairs based on collateral sensitivity can reduce or impede antibiotic resistance development. Also, we show that longitudinal population sequencing can identify putative epistatic effects (e.g. CIP Lineage B clonal interference in *gyrA* between mutant alleles Ser83del and Asp87Gly).

Using population amplicon sequencing allowed us to discover several mutations, numerate their frequency in the whole populations and follow their frequency over the experimental time course. However, we are unable to establish any subsidiary antibiotic mutations outside our gene loci or resolve any compensations mutations.

Nonetheless we believe these direct approaches will play a critical role in *in-vitro* adaptation evolution experiments and with methodology modification could extend to population sequencing studies *in-vivo.* This will be important in several cases including monitoring of chronic conditions that require long-term antibiotic treatment i.e. chronic urinary tract infections (Blango et al., 2014; Nolan et al., 2015) or cystic fibrosis patients colonized with *Pseudomonas aeruginosa* (Folkesson et al., 2012) and *Staphylococcus aureus* infections (Vanderhelst et al., 2012).

Author contribution

Author contribution for study conception and design: R.AH, C.M and M.O.A.S; acquisition of data: R.A.H; analysis and interpretation of data: R.A.H and C.M; drafting of manuscript: R.A.H; critical revision: R.A.H, C.M and M.O.A.S; funding of research: M.O.A.S

Funding

This research was funded by; EU H2020 ERC-20104-STG LimitMDR (638902) and the Danish Council for Independent Research Sapere Aude programme DFF -4004-00213. MOAS acknowledges additional funding from the Novo Nordisk Foundation and The Lundbeck Foundation.

Acknowledgments

We would like to thank Andreas Porse for developing the R growth script, describing the parameters and technical advice as well as the Bacterial Synthetic Group at the Novo Nordic Foundation Center for Biosustainability members for interesting scientific discussion to help stimulate our research and develop new ideas.

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Figure Legends

Figure 1. Experimental overview of investigation from sample collection description, adaptation laboratory evolution (ALE) overview and amplicon frequency work-flow. (**A**) Background to our frozen sample condition, showing 5 drug conditions (AMK, CHL, CIP, AMK-CHL and CHL-CIP) each with 3 lineages (Lineages A, B, C) covering 6 time-points (Day 0, 2, 4, 6, 8,10 and 12) from the adaptation laboratory experiments. (**B**)The workflow used to assess different loci from different drug conditions, lineages and time-points, where (1) DNA was extracted from the different sample, (2) each sample has amplicons for each locus these were generated by primers that had a DNA amplifying region and an Illumina Nextera XT compatible overhang, (3) these were then barcoded using indices from the Illumina Nextera XT library preparation kit, (4) all generated amplicons were pooled together based on fragment size and concentration, (5) the pooled amplicon

573 library was sequenced on MiSeq Illumina sequencing platform, (6) each sample
574 was sorted by barcode and each generated a file to map to the reference genome
575 and call variants in the bacterial population and calculate their frequency.
576
577 **Figure 2. Comparative cumulative mutation frequencies of gene loci for each**
578 **drug condition over deep-sequencing time-points** (**A**) The cumulative mutation
579 frequency calculated for each time-point, after removal of all mutations below 5%
580 (i) from left to right AMK (showing *cpxA*, *fusA* and *sbmA* gene loci), CHL (showing
581 *acrR*, *marR*, *rob* and *soxR* gene loci) and AMK-CHL (showing *acrR*, *cpxA*, *fusA*, *marR*,
582 *rob, sbmA* and *soxR* gene loci), (ii) from left to right CHL (showing *acrR*, *marR*, *rob*
583 and *soxR* gene loci), CIP (showing *acrR*, *gyrA*, *rob,* and *soxR* gene loci) and CHL-CIP
584 (showing *acrR*, *gyrA, marR*, *rob,* and *soxR* gene loci). (**B**) Heat-map of showing
585 difference cumulative mutational frequencies for *fusA* and *sbmA* gene loci from
586 AMK and AMK-CHL drug conditions at each time-point by Holm-Sidak's multiple
587 comparison two-way ANOVA between (All dark grey points are not significant and
588 all blue points are significant with a P-value below 0.0001).
589
590 **Figure 3. Epistatic mutation interaction observation on the whole bacterial**
591 **population.** Epistatic mutation interaction observation on the whole bacterial
592 population with the three types of mutational interactions shown with three
593 examples of each (**A**) Discordant mutational allelic interactions, where mutational
594 areas form different fractions of the population either red fill or grey fill, from left

595 to right (i) CIP-Lineage B (with *gyrA* Asp87Gly in grey line and fill and *gyrA*
596 Ser83del in red line), (ii) AMK-Lineage B (with *sbmA* Ala169Glu in grey line and
597 *cpxA* Trp184Arg in red line) and (iii)CHL-Lineage C (with *rob* Arg156His in grey
598 and *marR* Gln110* in red line) (**B**) Super-imposing mutational allelic interactions,
599 where both mutations are present at the same time therefore the mutational
600 frequency area is purple fill as it assumed that both mutations are co-existing in the
601 bacterial cells, from left to right, (i)AMK -Lineage C (with *cpxA* Ala79Val in grey line
602 and *fusA* Ala608Val red line), (ii) CHL-CIP Linage A (with *gyrA* Ser83del in grey line
603 and *marR* Val84Glu red line) and (iii) CIP Lineage A (with *gyrA* Ser83del in grey
604 line and *soxR* Ser126_Arg127delinsSer in red line) (**C**) Accumulating mutational
605 allelic interaction, where mutation arises the frequency area shown in grey fill,
606 then the second mutation arises in the background of the first therefore we assume
607 that both are present within the bacterial cells so purple frequency area fill was
608 used, from left to right (i)AMK –Lineage B (with *fusA* Pro610Gln in grey line and
609 *cpxA* Asp31Tyr in red line), (ii) CHL Linage B (with *marR* Arg86Trp in grey line
610 and *rob* Arg156His in red line) and (iii) CIP- Lineage A (with *gyrA* Asp87Tyr in
611 grey line and *gyrA* Gly75Ser in red line).
612
613 **Figure 4. Evaluation of strains based on relative growth rate and IC90 fold**
614 **change from ancestral wild-type, where green on the plots represent ≥ 1**
615 **relative growth rate and a IC90 fold change from wild type above ≥ 10 and**
616 **red ≤0.5 relative growth rate and a IC90 fold change from wild type above ≤2.**

(A) (i) Recombineed strains to assess the discordant mutation allelic interactions based on mutation found in CHL-Lineage C (strains labelled accordingly on the right hand-side) **(ii)** Recombineered strains to access the superimposing mutation allelic interactions based on mutation found in CHL-CIP Linage A (strains labelled accordingly on the right hand-side).

Supporting Information Legends

SI Table1. Primers for generation of amplicons

SI Table 2. Variant detection table

SI Table 3. Homologous recombineering oligos and verification primers

SI Figure 1. Coverage plots for each investigatory amplicon for AMK drug condition

SI Figure2. Coverage plots for each investigatory amplicon for CHL drug condition

SI Figure3. Coverage plots for each investigatory amplicon for CIP drug condition

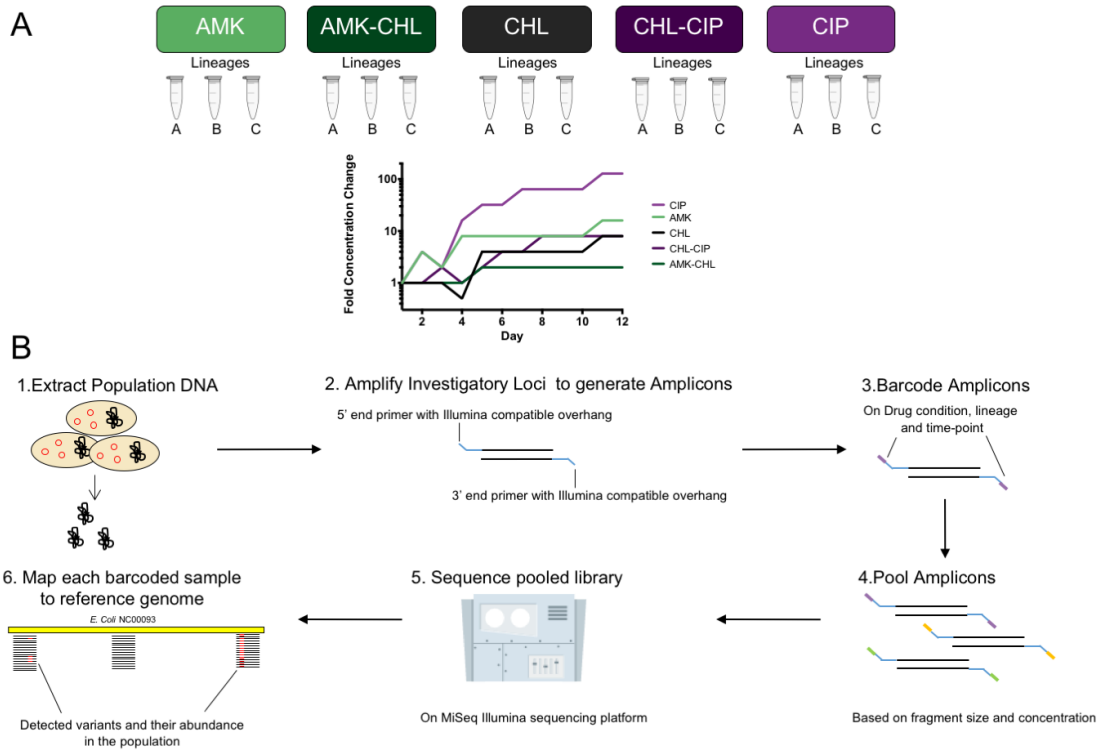
SI Figure 4. Coverage plots for each investigatory amplcion for AMK-CHL drug condition

SI Figure 5. Coverage plots for each investigatory amplcion for CHL-CIP drug condition

SI Figure 6. Significant variants tracking for each drug condition. Where mutations that appear at any given time-point at 30% or more are plotted. Mutations listed in black appear in all three lineages, those in dark grey appear in two lineages and those in light grey only appear in one lineage. (i)From left to right, AMK Lineage A (featuring mutations; *cpxA* (dark green fill): Asp31Tyr circle, Trp184Arg square, *fusA* (mid-dark green fill): Pro610Gln up-triangle, Pro610Thr down-triangle; *sbmA* (lightest green fill): asp194Asn hexagon with dot), B (featuring mutations *cpxA* (dark green fill): Asp31Tyr circle, Trp184Arg square; *fusA* (mid-dark green fill): Pro610Gln up-triangle, *sbmA* (lightest green fill): Asp194Glu circle with dot) and C (featuring mutations *cpxA* (dark green fill): Asp31Tyr diamond with dot, Trp184Arg square; *fusA* (mid-dark green fill): Ala608Val, Pro610Gln up-triangle, Pro610Thr down-triangle, Thr647Ala square with diagonal cross; *sbmA* (lightest green fill): Trp250* diamond with cross) (ii) From left to right, CHL Lineage A (featuring mutations; *rob* (mid-dark purple): Gly245Arg star), B (featuring mutations; *acrR* (light purple): Ala156Ser circle with diagonal cross; *marR* (mid-light purple): Arg86Trp square;

rob (mid-dark purple): Arg156His circle) and C (featuring mutations; *acrR* (light purple): Arg209fs hexagon; *marR* (mid-light purple): Arg86Trp square, Gln110* down-triangle; *rob* (mid-dark purple): Arg156His circle). (iii) From left to right, CIP Lineage A (featuring mutations; *gyrA* (mid-light green): asp87Tyr circle, Gly75Ser up-triangle, ser83del square; soxR (dark purple): Ser126_Arg127delinSer diamond with cross), B (featuring mutations; *gyrA* (mid-light green): asp87Gly diamond, ser83del square; *rob* (mid-dark purple): Arg156His hexagon with diagonal cross) and C (featuring mutations; *acrR* (light purple): Ser25* hexagon; *gyrA* (mid-light green): Gly81Cys circle with diagonal cross, *soxR* (dark purple): Arg127_Ser128delinsArg down-triangle). (iv) From left to right, AMK-CHL Lineage A (featuring mutations; *acrR* (light purple): Lys55Glu circle; *cpxA* (dark green): Ala187Thr square, Ile382_Thr383delinMetLeu up-triangle, Ser175Cys down-triangle; *soxR* (Dark purple): Ala18Glu hexagon, glu150fs square with diagonal cross), B (A (featuring mutations; *acrR* (light purple): Asp157Asn star, Cys205Phe square, Lys55Glu circle and Met175Arg) and C (featuring *acrR* (light purple): Ala156Ser diamond with Ala156Ser, Arg209fs square with diagonal cross, Lys55Glu; *cpxA* (dark green): Ala187Thr and soxR (dark purple) Ala18Glu hexagon). (V) From left to right, CHL-CIP Lineage A (featuring mutations gyrA (mid-light green): Phe96fs circle, Ser83del square; marR (mid-light purple): Cys51Ser half-dark up-triangle, Val84Glu down triangle; *soxR* (dark purple): Arg20Cys square) B (featuring mutations *acrR* (light purple): Cys205delinsCysArg circle, Thr5Ala circle half dark; *gyrA* (mid-light green): Ser83del square; *soxR* (dark

purple): Arg20Cys square, Ser126_Arg127delinsCys diamond) and C (featuring mutations *acrR* (light purple): Cys205delinsCysArg circle; *gyrA* (mid-light green): Ser83del square; *soxR* (dark purple): Arg20Cys square, Ser126_Arg127delinsSer up-triangle).


A
AMK
AMK-CHL
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Lineages
A B C
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100
10
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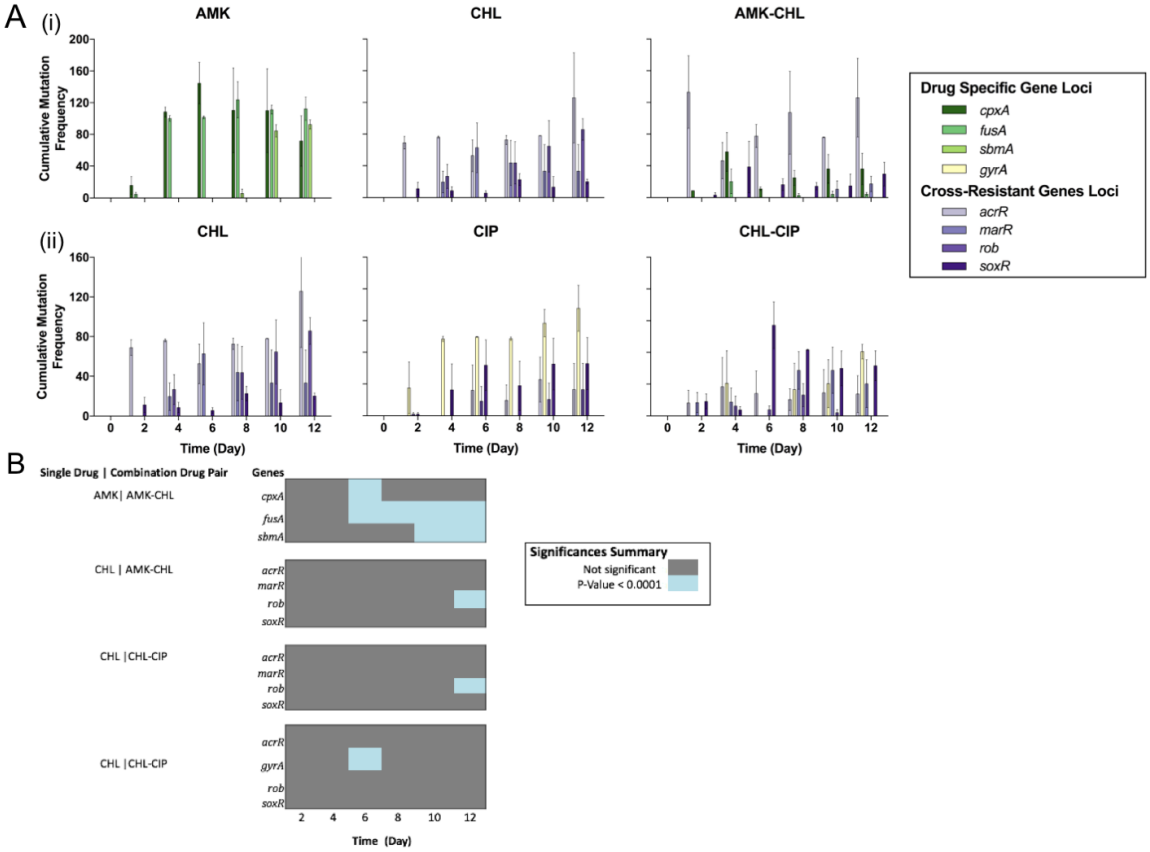
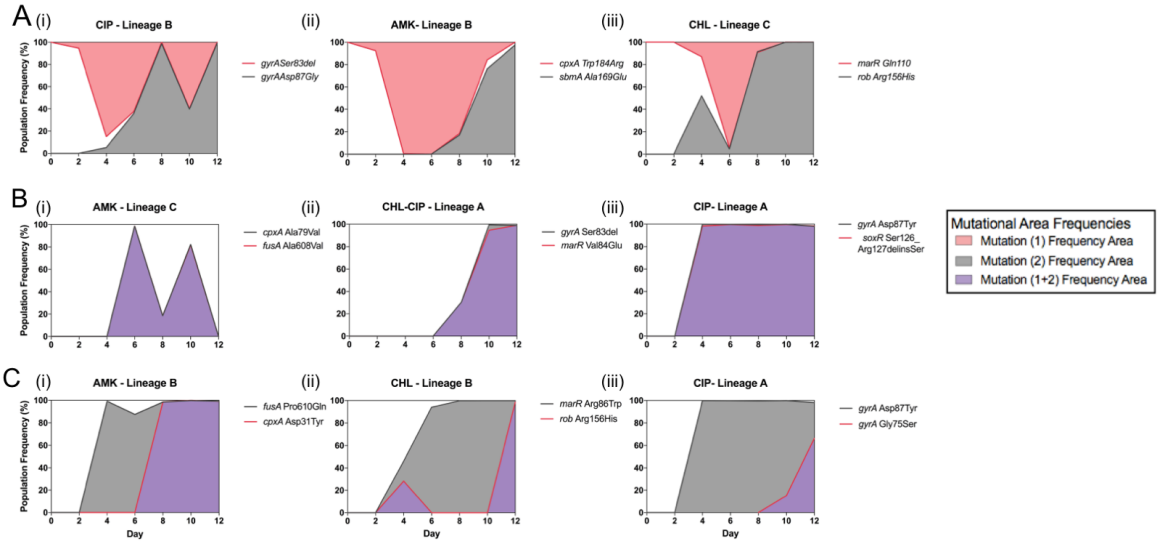
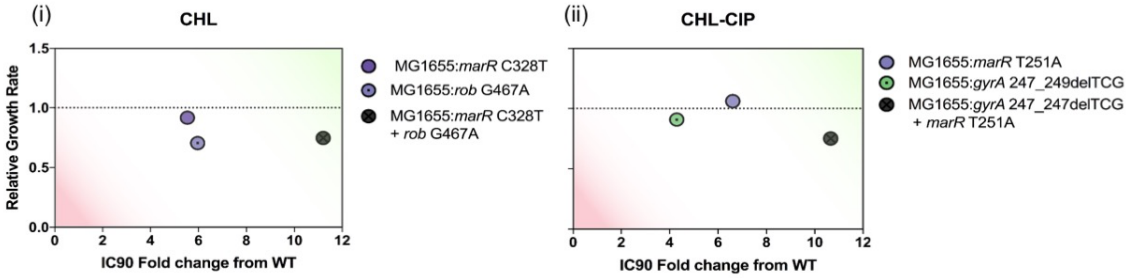
B
1.Extract Population DNA
2. Amplify Investigatory Loci to generate Amplicons
5' end primer with Illumina compatible overhang
3' end primer with Illumina compatible overhang
3.Barcode Amplicons
On Drug condition, lineage and time-point
4.Pool Amplicons
Based on fragment size and concentration
5. Sequence pooled library
On MiSeq Illumina sequencing platform
6. Map each barcoded sample to reference genome
E. Coli NC00093
Detected variants and their abundance in the population


A
(i)
AMK
CHL
AMK-CHL
Cumulative Mutation Frequency
(ii)
CHL
CIP
CHL-CIP
Time (Day)
Drug Specific Gene Loci
cpxA
fusA
sbmA
gyrA
Cross-Resistant Genes Loci
acrR
marR
rob
soxR
B
Single Drug | Combination Drug Pair
Genes
AMK| AMK-CHL
CHL | AMK-CHL
CHL |CHL-CIP
CHL |CHL-CIP
Significances Summary
Not significant
P-Value < 0.0001
Time (Day)


A
(i)
CIP - Lineage B
gyrASer83del
gyrAAsp87Gly
(ii)
AMK- Lineage B
cpxA Trp184Arg
sbmA Ala169Glu
(iii)
CHL - Lineage C
marR Gln110
rob Arg156His
B
(i)
AMK - Lineage C
cpxA Ala79Val
fusA Ala608Val
(ii)
CHL-CIP - Lineage A
gyrA Ser83del
marR Val84Glu
(iii)
CIP- Lineage A
gyrA Asp87Tyr
soxR Ser126_ Arg127delinsSer
C
(i)
AMK - Lineage B
fusA Pro610Gln
cpxA Asp31Tyr
(ii)
CHL - Lineage B
marR Arg86Trp
rob Arg156His
(iii)
CIP- Lineage A
gyrA Asp87Tyr
gyrA Gly75Ser
Population Frequency (%)
Day
Mutational Area Frequencies
Mutation (1) Frequency Area
Mutation (2) Frequency Area
Mutation (1+2) Frequency Area


(i)
CHL
Relative Growth Rate
1.5
1.0
0.5
0.0
0
2
4
6
8
10
12
IC90 Fold change from WT
MG1655:marR C328T
MG1655:rob G467A
MG1655:marR C328T
+ rob G467A
(ii)
CHL-CIP
0
2
4
6
8
10
12
IC90 Fold change from WT
MG1655:marR T251A
MG1655:gyrA 247_249delTCG
MG1655:gyrA 247_247delTCG
+ marR T251A

Manuscript II: Direct sequencing of *Pseudomonas aeruginosa* from sputum of cystic fibrosis patients undergoing antimicrobial therapy

Direct sequencing of *Pseudomonas aeruginosa* from sputum of cystic fibrosis patients undergoing antimicrobial therapy

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Abstract

Cystic fibrosis (CF) patients due to inherited genomic mutations suffer from dehydrated mucous that provide the perfect matrix to support opportunistic pathogens. This is of critical concern in the lungs where infections can easily occur and debilitate lung function. Antimicrobial therapy has been good at delaying chronic lung infections but despite best efforts, patients often become chronically infected with chronic *Pseudomonas aeruginosa* in their lungs. Aggressive antimicrobial treatment helps to lower the *P. aerugionsa* bacteria population to restore better lung function, but over-time *P. aerugionsa* evolve to become antibiotic resistant and the therapy become less effective overtime. This study addresses CF patients that are inflicted with chronic *Pseudomonas aeruginosa* lung infections by directly analyzing their by directly analyzing their sputum during hospitalization for antimicrobial therapy. This is first study to directly analyze un-perturbed sputum samples in 19 known antibiotic resistance and patho-adaptive genes (*ampC, ampDh3, fusA1, fusA2, gyrA, gyrB,* mexR, *mexS, mexT, mexZ, nfxB, opr86, phoP, PhoQ, pmrA, pmrB and rpoB*) to understand abundance of individual mutational variants and mutational variant populations effects. By using whole gene sequencing directly on sputum samples from CF patient we yield important results regarding *P. aeruginosa* lung population, which could help optimizing antibiotic treatments and reduce antibiotic resistance development in persistence and chronically *P. aeruginosa* infected CF patients.

Key word: *Pseudomonas aeruginosa*, cystic fibrosis, direct whole gene population sequencing, sputum, and clonal interference

Introduction

Cystic fibrosis (CF) is an autosomal recessively inherited disease that affects about 7 in 100,000 people in the EU countries (Farrell, 2008). It is caused by a mutation in the CFTR protein that function as a chloride channels causing viscous dehydrate mucous on muco-epithial linings, which is difficult to expel. Un-expelled mucus provides the perfect habitat for a consortium of opportunistic microorganisms (Tang et al., 2014) and consequently lung infections are the major cause of morbidity and mortality in CF patients. Therefore, it is routine for CF patients to have recurrent antimicrobial therapy to treat the lung infections and to maintain lung function (Johansen et al., 2004). Antimicrobial therapy has help to delay the development of chronic infections from infancy to early adolescents ages (Döring and Hoiby, 2004; Frederiksen et al., 1997; Valerius et al., 1991). However, CF patient often become infected with ubiquitous environmental bacterial pathogen, especially, *Pseudomonas aeruginosa,* which often becomes established as a chronic infection.

Many studies focus on the adaptive process of *P. aeruginosa* to becoming an established chronic infection (Marvig et al., 2015; Smith et al., 2006; Sommer et al.,

2016) or developing resistance against antimicrobial therapy (Feng et al., 2016; Jochumsen et al., 2016).

Here we present an *in-vivo* molecular study, following four CF patients hospitalized for antimicrobial treatment. By directly extracting the DNA from the sputum from three time-points at day 0, 7, and 14; we can observe the un-biased mutational variants that are occur within the genes by using our molecular pipeline (Figure 1). Our pipeline, unlike other proceeding studies analyzes the whole gene, by PCR amplification up and downstream of our genes of interest. With the information, we can access the amount of mutated alleles occurring at each time point and state what translational effect they cause. By understanding the population dynamics and mutational alleles that are enriched during antimicrobial therapy we can acquire knowledge about bacterial populations that can be targeted in CF patients with chronic *P. aeruginosa* infections. Future application for CF patients from this study are: deployment of antimicrobial treatment that reduced the evolution of antimicrobial resistance; and for CF patients that suffer from persistent *P. aeruginosa* infections, with the motivation how to delay chronic colonization.

Material and methods

Patient

Our study consisted of 4 males CF patients undergoing antimicrobial treatment at the CF clinic at Rigshospitalet, Copenhagen, Denmark. The age of the patients ranged from 26 to 53 years, all were diagnosed with chronic *P. aeruginosa* infection in accordance to the Copenhagen criteria (Johansen et al., 2004). Patient's treatment at the hospital is recorded in (Table 1). Samples were stored at -20 after collection, until DNA extraction was performed.

Microbiology

All sputum samples were Gram-stained and microscopically evaluated and cultured aerobically on selective agar media. These media include a Sabouraud plate, a 7% NaCl plate, a *B. cepacia* plate containing colistin and gentamicin and a "blue plate" (modified Conradi Drigalski's medium) selective for Gram-negative rods and non-selective media including 5% Danish blood agar and chocolate agar. Direct plating of sputum samples on a 14-cm blood agar plate (State Serum Institute, Copenhagen, Denmark) with discs containing anti-pseudomonas antibiotics including colistin, tobramycin, meropenem, ciprofloxacin, aztreonam, piperacillin/tazobactam and ceftazidime. Susceptibility testing of the sputum samples was done by the agar diffusion method on Danish blood agar plates (Statens Serum Institut, Copenhagen, Denmark), employing Neosensitabs® (Rosco, Roskilde, Denmark)(Ciofu et al., 1996). Isolated bacteria were identified as described previously (Johansen et al., 2008) Before 2011, biochemical profiling of *P. aeruginosa* was based on API 20NE (bioMérieux), and from 2011 on MALDI-TOF mass spectrometry (Bruker, Germany).

DNA extraction from sputum samples

All thawed sputum samples were treated with 30μL (1M) tris (2-carboxyethyl) phosphine, and 10μL proteinase K (20mg/mL) (ThermoFisher Scientifc) and 1mL of DNA/RNA shield (Zymo Research) and vortexed for 30 seconds. Samples were then added to a 2mL impact resistant screw-top tube with 300mL zirconia/glassbeads with a diameter 0.1mM (Carl Roth International) and vortexed on a secure and horizontal holder at maximum speed for 5 mins. Then genomic DNA was extracted from all sputum samples by ZR –Duet RNA/DNA mini-prep kit (Zymo Research) and done in accordance to the kits recommendations.

Primer Design

The genes selected were chosen based on knowledge of their involvement *de-novo* antibiotic resistance. The following genes of the *P. aeruginosa* core genome were selected for sequencing: *ampC, ampDh3, ftsI*, *fusA1, fusA2, gyrA, gyrB mexS, mexR, mexT, mexZ, nfxB, opr86, parC, pmrA, pmrB, phoQ, phoR, rpoB.* Primers were designed to amplify 19 genes associated with antibiotic susceptibility (**Supplemental Fig. 1**). All primers were tested with genomic DNA from *P. aeruginosa* strains: PAO1, DK2 and PA14 the sequences of the 19 target genes were extracted from the Pseudomonas database (Winsor et al., 2016). PCR products were tested with PAO1

and DK-2 DNA extracts verified by 1% agarose gel electrophoresis with GelRed (Biotium).

Whole gene library preparation and sequencing

All samples were amplified using the 19 primer pairs in (**Supplemental table 1**), PCRs from sample DNA extracts were done independently to each other to avoid cross contamination. Each PCR reaction was done in 0.2mL sterile PCR tubes and consists of 10 μ L Phusion High-Fidelity PCR master mix (Thermo Fischer Scientific), 0.5 μM forward primer, 0.5 μ M reverse primer, 1 μL DNA template and H_20 to a total reaction volume of 20 μL. The PCR amplification used an initial denaturation of for 98^{o}C 30 seconds, 30 cycles consisting of a 98^{o}C for 10 seconds denaturation, 60^{o}C for 10 seconds annealing, 72^{o}C for 3 min elongation, 72^{o}C final extension for 180 seconds then hold at 4^{o}C. To validate correct amplification gel electrophoresis was performed with a 96-well 2% E-gel with SYBR safe DNA gel stain (Invitrogen) on the E-base electrophoresis device for 13 min (Invitrogen) and gel images analyzed on the E-editor 2.02 software (Invitrogen). Each sample was cleaned with 1.8x Ampure bead and done in accordance an online protocol (Beckman Coulter 2013). The 19 PCR reactions for each sample DNA extract were pooled together; each pooled PCR product in accordance to its original sample. 200ng of pooled DNA in a volume of 130μL was sheared in a Covaris focused-ultrasonicator E220, for 55 seconds with a peak incidence power of 105W, duty factor 5% and cycles per burst 200. Sheared pooled DNA samples were then prepared for sequencing by the Kapa

Hyper Plus DNA library preparation kit (Kapa Biosystems) and barcoded using LT-Truseq single-index adapter kit (Illumina Inc). Barcoded libraries were measured for DNA concentration with Qubit dsDNA HS Assay kits and measured on Qubit Fluorometer (Thermo Fischer Scientific Inc 2015) and average fragment size with Fragment analyzer (Advanced Analytical Technologies, Inc). Barcoded libraries were pooled from fragment size and DNA concentration values to form a sequencing library based. The sequencing library was sequenced on the MiSeq platform with Miseq reagent kit V3, sequence read length 2x300bp (Illumina Inc).

Sequence Analysis

All raw sequencing read from the gene sequencing has been deposited in the Sequence Read Archive (SRA) under bioproject accession number xxxx. Sequencing reads were mapped to the reference genome (GenBank accession AE004091) using CLC genomics workbench version 8.5.1(Qiagen). To verify sequencing coverage, mapping read files from CLC genomic workbench data was analyzed in Excel and plots designed in Prism 6 (GraphPad Software Inc). To ensure good coverage our threshold for average coverage was set at 100, samples that had low coverage were re-read until sufficient coverage was achieved (**Supplemental Fig 1-4**). Variants were detected by using the low frequency variant detection calling function in CLC genomic workbench V 8.5.1 (Qiagen), provide all the variants that had a population frequency of ≥1%. (**Supplemental Table 2**).

Results

The study consisted of 4 CF patients chronically with *P. aerugionsa* with an age range from26 to 53 years. Each patient provided three sputum samples on day 0, 7 and 14 during hospitalized antimicrobial treatment, minus one patient who was unable to provide a day 7 cough sample. Following our pipeline we extracted DNA from each of the samples and using our pipeline (Figure 1) the intrinsic antimicrobial resistance development was assessed in 19 genes that have been previously reported to confer resistance against certain classes of antibiotics. These were: for aminoglycoside (genes of interest were: *fusA1* (Marvig et al., 2013), *fusA2* (Marvig et al., 2013), and rpoB (Qi et al., 2014)); for peptidoglycan synthesis inhibiting classes (genes of interest were: *ampC* (Marvig et al., 2013) , *ampDh3* (Lee et al., 2013) and *ftsI* (Smith et al., 2006)); for fluoroquinolone (genes of interest were: *gyrA* (Feng et al., 2016; Marvig et al., 2013), *gyrB* (Feng et al., 2016; Marvig et al., 2013), *parC* (Feng et al., 2016) and *rpoB* (Pietsch et al., 2017)); polymixin (genes of interest were: *opr86* (Jochumsen et al., 2016), *phoP* (Smith et al., 2006) *phoQ* (Gutu et al., 2013; Moskowitz et al., 2012), *pmrA* (Moskowitz et al., 2012) and *pmrB* (Moskowitz et al., 2012; Smith et al., 2006)); and patho-adaptive genes that confer cross-resistance (genes of interest were: *mexR* (Vestergaard et al., 2016), *mexS*(Richardot et al., 2016), *mexT* (Smith et al., 2006), *mexZ* (Smith et al., 2006) and *nfxB* (Monti et al., 2013)).

188 Only two patients provide a day 0 and day 14 samples for antimicrobial
189 susceptibility testing samples to the department of clinical microbiology
190 department, these were patient CF156 and CF83 (Table 2). From the antimicrobial
191 susceptibility testing we observed phenotypic changes before and after in both
192 patients. For CF156 in the non-mucoid *P. aeruginosa* we saw changes in: colistin,
193 from susceptible to resistant; imipenem, from susceptible to intermediate; and
194 meropenem, and from intermediate to resistant. In the same patient we also saw in
195 the mucoid *P. aeruginosa* sample a phenotypic change of impenem from day 0 being
196 intermediate to susceptible on day 14. Whereas for patient CF83 in the mucoid *P.*
197 *aeruginosa* antimicrobial susceptible typing results we saw changes in:
198 ciprofloxacin, from susceptible to intermediate; and imipenem, from susceptible to
199 intermediate. The overall observation from these two patients is that the use
200 antimicrobial therapy leads to induction of resistance to several antibiotics. This is
201 not surprising as antimicrobials are well know for selecting pre-existing and *de-novo*
202 mutants (Andersson and Hughes, 2014).

203

204 **Detection of mutational variants reveal the high abundance of**
205 **mutagenic events of *P. aeruginosa* in the CF lung**

206 Following coverage analysis, where all samples had to meet the requirements of all
207 genes having an average coverage of 100 sequencing reads per base in the open
208 reading frame of the gene, the variant detection and analysis was performed. From
209 our results we able to plot an overview of all the variant types detected with a
210 population frequency ≥5% for each patient and time-point (Figure 2). From these
211 plots, we found that the maximum total individual variant detected were was in

patient CF382 at day 0 with 514 different variants detected and the minimum individual variant detected in patient CF83 at day 0 with 143 different variants detected. Most of the detected variants were synonymous mutations, the second most abundant mutation type was non- synonymous SNPs, followed by the third most abundant mutation type being frameshift mutations and the fourth most abundant mutation type was premature stop mutations where few to no mutations were detected. Similar distribution of mutational variant types from amplicon sequenced *P. aerugionsa* isolates from the CF lung have been reported by Grieple et al. 2016.

There was also a general trend amongst three of the patients that the amount of individual mutation variants detected increased during the 14-day antimicrobial treatment. This was highest in patient CF83 where the individual mutational variants for day 0, 7 and 14 were: 143; 173; and 233 (Figure 2 (iv)). In patient CF243 the individual mutational variants detected for day 0, 7 and 14 were: 253; 204; and 305 (Figure 2 (iii)). In patient CF243 the individual mutational variants detected for day 0, 7 and 14 were: 175; 156; and 201 (Figure 2 (ii)). An exception to this trend was patient CF382, where the individual mutational variants detected for day 0 and 14 were 514 and 158 (Figure 2 (i)). These results imply that when patients are receiving antimicrobial therapy the *P. aeruginosa* bacterial population in the lung experiences possibly an expansion in genomic diversity. Whether this is a direct or downstream effect of antibiotics, such as the production of reactive oxygen species (Kohanski et al., 2007) or by antibiotics inducing the quorum sensing mechanisms endogenous to *P. aeruginosa* that can have pleiotropic effects (Skindersoe et al., 2008) need to be further elucidated.

Evaluation of non-synonymous mutations during antimicrobial treatment show mutational enrichment and clonal inference within the bacterial population

We then further analyzed our data by evaluating the non-synonymous mutations, we were interested to follow the trajectories of mutations that were enriched and un-selected for during antimicrobial therapy (Figure 3). To achieve this we examined the non-synonymous mutations that were present at a least one time-point ≥5%. In this figure, we could see in all patients that there was a clear enrichment of mutagenic allelic variants over the treatment time period (Figure 3A (i-iv)). We were interested if these mutations had been previously detected and if there was any information regarding these mutations conferring antibiotic resistance. From the 41 non- synonymous mutations, 11 of these have been reported in literature, with 3 mutations all from patient CF382 with results confirming that these mutations confer resistance (Table 3). Our molecular pipeline detected several mutations that have not previously been reported, this could be due to our direct DNA extraction, amplification and sequencing, capturing all present variants.

From the enriched mutation variants, we could see that each patient had a unique response in the individual variants that were produced. This could be a result of the historical contingency of the *P. aeruginosa* bacteria in the lung, the clone-type they were infected with and the treatment used (Table 1). Patient CF382 is known to be

infected with a hypermutator strain, and was treated with drug classes: aminoglycosides, cephalosporin, fluoroquinolone and polymixins. Therefore, it was not surprising we saw enriched mutations in the following genes: *ampC, ampDh3, fusA1, fusA2, gyrA, gyrB, mexZ, opr86, phoQ, pmrA, pmrB* and *rpoB.* For CF156, the patient was treated with polymixin and fluoroquinole classes of antibiotics. This patient had enriched mutations in: *ampC, ampD3h, mexT, opr86, pmrA* and *rpoB*. The *ampC* and *ampD3h* mutations that were enriched during treatment were an anomaly we didn't expect to observe, as the *ampC* and *ampDh3* mutations we expect to confer beta-lactam, cephalosporin and carbapenem resistance. We suspect that these new mutants in *mexT, opr86, pmrA* and *rpoB* that appeared had *ampC* and *ampDh3* mutations in their genomic background, and were selected as a result of genomic hitchhiking a common phenomenon often seen in adaption laboratory evolution experiments (Elena and Lenski, 2003). Patient CF243 was treatment with aminoglycosides, carbapenem, fluoroquinolone and polymixin classes of antibiotics. The high levels of enrichment consisted in mutations in *pmrB* gene that is known to confer resistance to polymixins (Moskowitz et al., 2012; Owusu-Anim and Kwon, 2012; Schurek et al., 2009) and *mexT,* which is known to confer cross-resistance by the expression of efflux pumps (Alyaseen et al., 2005). Patient CF83 was treated with aminoglycosides, cephloasporin, fluoroquinolones and polymixins. High frequency enrichment was found in *rpoB*, *mexT*, and *ftsI* and low-level enrichment in *mexS* and *gyrA*. Where the rpoB mutants confer aminoglycoside resistance (Qi et al., 2014) and ciprofloxacin (Pietsch et al., 2017), *ftsI* mutants confer resistance against

carbapenems (Cabot et al.), and *mexT* mutants provide general cross-resistance (Alyaseen et al., 2005).

We also observed strong clonal interference is exhibited in two patients, CF382 and CF83 (Figure 3A (i) and (iv), 3B (i) and (iv)), with marginal clonal interference in CF156 and CF243 (Figure 3A (ii) and (iii), 3B (i) and (iv)). In all patients, we see that there are sub-populations of bacterial mutational variants, a trait also seen by others regarding sub-populations of virulent *P. aeruginosa* in the CF lung (O'Brien et al 2017). Therefore, establishment of novel mutations seems to readily occur, but fixation in the population is a rare event, unlike *in-vitro* adaptation evolution experiments in other Gram-negative species (Wong and Seguin, 2015). This could be an artifact of spatial structural and heterogeneity in the CF lung (Folkesson et al., 2012; Markussen et al., 2014; Winstanley et al., 2016) or the short time period our study was preformed in. Thus, showing the complex bacterial population dynamics within CF patients.

Discussion

Within this study we use whole gene population sequencing method to study individual mutation variant events and mutagenic populations dynamics. Our approach was to extract the DNA from the patient sputum samples, amplify genes of interest and sequence with no *P. aerugionsa* isolation cultivation steps. With the

303 development and growing accessibility of next generation sequencing platforms,
304 studies analyzing evolution in bacterial populations are growing. In particular, *P.*
305 *aerugionsa* population sequencing following cultivation from CF patient samples
306 (Fischer et al., 2016; Greipel et al., 2016; O'Brien et al., 2017).

307

308 In CF patients that are chronically infected with *P. aeruginosa*, we have observed the
309 vast array of mutant clones occupy one environment, a phenomenon also described
310 by Greiple et al 2016. Yet by following events longitudinally we can observe how
311 these compete by clonal interference to establish the clone types that best aids
312 population survival.

313

314 The bacterial populations we study also display similar characteristics that have
315 been reported in-vitro adaptation studies with other Gram-negative bacterial
316 species, such as clonal interference and multiple mutant enrichment (Fogle et al.,
317 2008; Gerrish and Lenski, 1998; Sniegowski et al., 1997; Wong and Seguin, 2015).

318

319 Our results are important but limited therefore in future we would like to examine a
320 larger cohort of chronically infected patient and for longer periods of time. This will
321 allow us to examine key factors such as: which antibiotics treatment cause the
322 highest and lowest generation of mutational variants; and by studying population

dynamics for longer periods of time can we capture clonal fixation events and the implications of these population fixations by *in-vitro* experimentation.

Therefore, our long-term version is to optimize antimicrobial treatment in chronically infected CF patients with *P. aeruginosa* and in patients that have persistent colonization and find strategies to delay the chronic establishment of *P. aeruginosa*.

Acknowledgements

The CF patients and nurses at the adult CF center at Rigshospitalet are thanked for their collaboration in this project. We would also like to thank Anna Koza from CFB's next generation sequencing laboratory for her technical support and advice. HKJ was supported by the Novo Nordisk Foundation as a clinical research stipend and Rigshospitalet Rammebevilling 2015-17 and Lundbeckfonden Grant R167-2013-15229. MOAS funding by, EU H2020 ERC-20104-STG LimitMDR (638902) and the Danish Council for Independent Research Sapere Aude programme DFF -4004-00213 additional funding from the Novo Nordisk Foundation and The Lundbeck Foundation

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Figure Legends

Figure 1. Methodology workflow overview (1) Thaw collected sputum samples, (2) Extract DNA from each sample (3) PCR all 19 genes for each DNA extract and verify PCR products (4) Shear DNA by sonication for DNA fragments ca. 600bp (5) Prepare sequencing library by kit and barcode then pool, (6) Sequence Miseq Illumina platform.

Figure 2. Overview of all variant types detected with a population frequency ≥5% throughout treatment course for all genes combined (i) Patient CF382, (ii) CF156, (iii) CF243 and (iv) CF83. Variant types correspond to key on the right.

Figure 3. Enriched and unselected variant alleles throughout treatment for each patient (A) Enriched variant alleles during treatment (i) Patient CF382, (ii) CF156, (iii) CF243 and (iv) CF83. variant types correspond to key on the right. **(B)** Unselected variant alleles during treatment (i) Patient CF382, (ii) CF156, (iii) CF243 and (iv) CF83. Each variant is listed on the right of each plot.

Table 1. Patient history

Patient	Daily treatment over hospitalization period	*P. aeruginosa* Clone Type	Hypermutator strain
CF382	Intravenous 3g Ceftazidime 3g x 3 Intravenous 500 mg Tobramycin x 1 Inhalation Promixin 3 mio IE x 1 Oral 750 mg Ciprofloxacin x 2 Oral 250 mg Azithromycin x 1	DK32	Yes
CF156	Oral 750 mg Ciprofloxacin x 2 Intravenous 9 mio IE Colistin x1	DK1 and DK2	No
CF243	Intravenous 9 mio IE Colistin x1 Intravenous 2g Meropenem x 2 Inhalation 240mg Ciprofloxacin x 2 Inhalation 300mg Tobramycin x 2 Oral 250 mg Azithromycin x 1	DK2	No
CF83	Inhalation Promixin 3 mio IE x 1 Intravenous 3g Ceftazidime 3g x 3 Intravenous 500mg Tobramycin x 1 Inhalation Promixin 3 mio IE x 1 Oral 750mg Ciprofloxacin x 2 Oral 250mg Azithromycin x 1	Unknown	No

558 **Table 2.** Antimicrobial susceptibility testing of each of patient
559

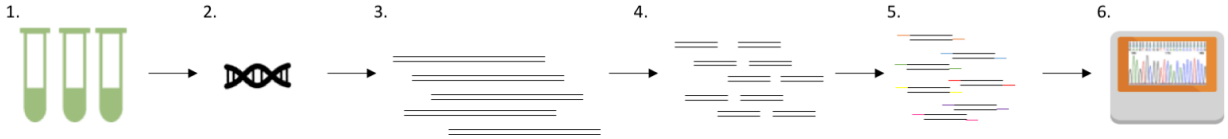
Patient	Time-point	*P. aeruginosa* type	Antimicrobial	Antimicrobial susceptibility type
CF382	Day 0	Mucoid	Aztreonam	R
			Ceftazidime	I
			Ciprofloxacin	S
			Colistin	S
			Imipenem	R
			Meropenem	R
			Moxifloxacin	R
			Piperacillin + Tazobactam	I
			Tobramycin	S
		Non-Mucoid	Aztreonam	R
			Ceftazidime	I
			Ciprofloxacin	S
			Colistin	S
			Imipenem	R
			Meropenem	I
			Moxifloxacin	R
			Piperacillin + Tazobactam	I
			Tobramycin	S
CF156	Day 0	Mucoid	Aztreonam	R
			Ceftazidime	R
			Ciprofloxacin	S
			Colistin	S
			Imipenem	I
			Meropenem	I
			Moxifloxacin	R
			Piperacillin + Tazobactam	I
			Tobramycin	S
		Non-mucoid	Aztreonam	R
			Ceftazidime	R
			Ciprofloxacin	S
			Colistin	S
			Imipenem	S
			Meropenem	I
			Moxifloxacin	R
			Piperacillin + Tazobactam	R
			Tobramycin	I
	Day 14	Mucoid	Aztreonam	R
			Ceftazidime	R
			Ciprofloxacin	S

			Colistin	S
			Imipenem	S
			Meropenem	I
			Moxifloxacin	R
			Piperacillin + Tazobactam	I
			Tobramycin	S
		Non-mucoid	Aztreonam	R
			Ceftazidime	R
			Ciprofloxacin	S
			Colistin	R
			Imipenem	I
			Meropenem	R
			Moxifloxacin	R
			Piperacillin + Tazobactam	R
			Tobramycin	S
CF243	Day 0	Non-mucoid	Aztreonam	R
			Ceftazidime	R
			Ciprofloxacin	R
			Colistin	S
			Imipenem	R
			Meropenem	R
			Moxifloxacin	R
			Piperacillin + Tazobactam	I
			Tobramycin	R
CF83	Day 0	Mucoid	Aztreonam	S
			Ceftazidime	S
			Ciprofloxacin	S
			Colistin	S
			Imipenem	S
			Meropenem	S
			Moxifloxacin	R
			Piperacillin + Tazobactam	S
			Tobramycin	S
	Day 14	Mucoid	Aztreonam	S
			Ceftazidime	S
			Ciprofloxacin	I
			Colistin	S
			Imipenem	I
			Meropenem	S
			Moxifloxacin	R
			Piperacillin + Tazobactam	S
			Tobramycin	S

562 **Table 3.** Enriched non-synonymous mutations that are reported in literature

Patient	Gene	Amino Acid Change	Literature	Reported MIC increase
CF382	*ampC*	Thr105Ala	(Rodríguez-Martínez et al., 2009)	Cefapime ->64 fold Cefepime ->8 fold Imipenem -> 10 fold Meropenem -> 2 fold
CF83	*ftsI*	Arg503Cys	(Cabot et al., 2016)	
CF382	*fusA2*	Gln610His	(Greipel et al., 2016)	
CF382	*gyrA*	Ala51Val	(Greipel et al., 2016)	
CF382	*gyrB*	Ser466Phe	(Bruchmann et al., 2013) (Llanes et al., 2011)	Ciprofloxacin-> 10 fold
CF382	*pmrA*	Leu71Arg	(Bezuidt et al., 2013) (Schurek et al., 2009)	
CF382	*pmrB*	Ala282Thr	(Moskowitz et al., 2012)	Colistin-> 8 fold
CF382	*pmrB*	Tyr345His	(Owusu-Anim and Kwon, 2012)	
CF83	*rpoB*	Arg1038His	(Greipel et al., 2016)	

563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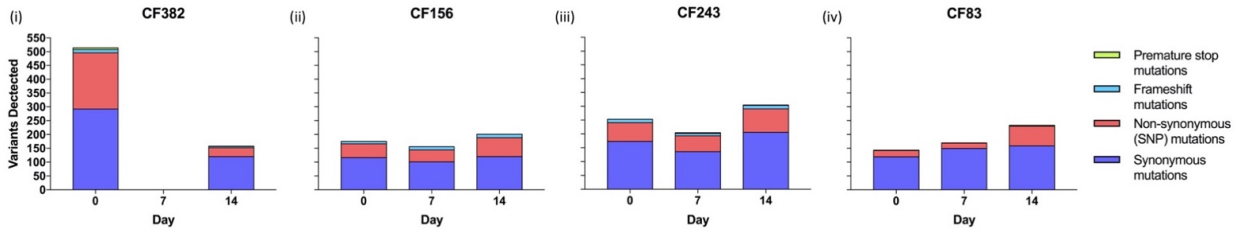
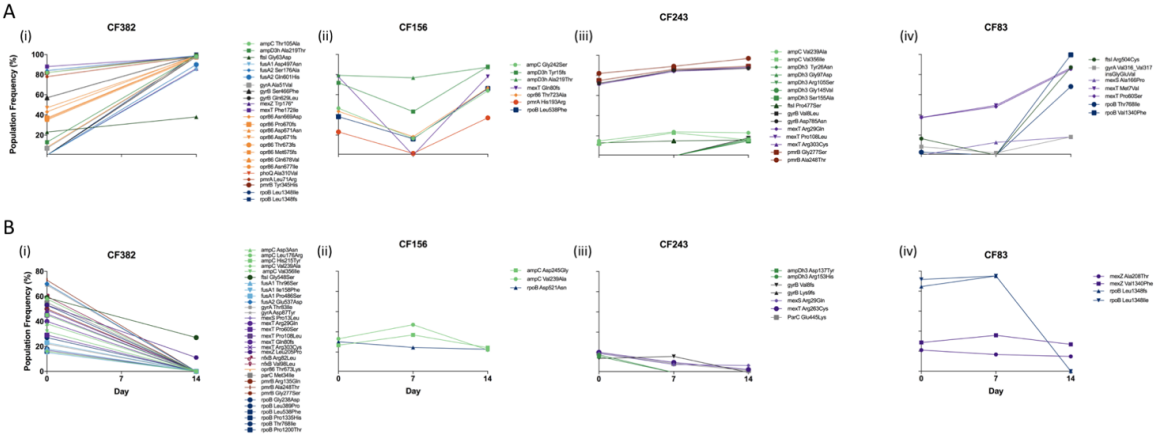

(i)
CF382
(ii)
CF156
(iii)
CF243
(iv)
CF83
Variants Dectected
550
500
450
400
350
300
250
200
150
100
50
0
0
7
14
Day
Premature stop mutations
Frameshift mutations
Non-synonymous (SNP) mutations
Synonymous mutations


A
(i)
CF382
Population Frequency (%)
(ii)
CF156
(iii)
CF243
(iv)
CF83
B
(i)
CF382
Population Frequency (%)
Day
(ii)
CF156
Day
(iii)
CF243
Day
(iv)
CF83
Day